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(21) International Application Number: PCT/US00/07133 (22) International Filing Date: 17 March 2000 (17.03.2000) (30) Priority Data: 60/125,251 19 March 1999 (19.03.1999) US (60) Parent Application or Grant CORNELL RESEARCH FOUNDATION, INC. [/]; () LOUISIANA STATE UNIVERSITY [/]; () PURDUE RESEARCH FOUNDATION [/]; () BARANY, Francis [/]; () DAY, Joseph, P. [/]; () HAMMER, Robert, P. [/]; () BERGSTROM, Donald, E. [/]; () GOLDMAN, Michael, L.; ().	Published	
(54) Title: COUPLED POLYMERASE CHAIN REACTION-RESTRICTION ENDONUCLEASE DIGESTION-LIGASE DETECTION REACTION PROCESS (54) Titre: REACTION EN CHAÎNE DE LA POLYMERASE COUPLEE, DIGESTION DE L'ENDONUCLEASE DE RESTRICTION, ET PROCÉDE DE REACTION DE DETECTION PAR LIGASE		
(57) Abstract <p>The present invention provides a method for identifying one or more low abundance sequences differing by one or more single-base changes, insertions, or deletions, from a high abundance sequence in a plurality of target nucleotide sequences. The high abundance wild-type sequence is selectively removed using high fidelity polymerase chain reaction analog conversion, facilitated by optimal buffer conditions, to create a restriction endonuclease site in the high abundance wild-type gene, but not in the low abundance mutant gene. This allows for digestion of the high abundance DNA. Subsequently the low abundant mutant DNA is amplified and detected by the ligase detection reaction assay. The present invention also relates to a kit for carrying out this procedure.</p>		
(57) Abrégé <p>L'invention concerne un procédé d'identification, dans un groupe de séquences nucléotidiques cibles, d'une ou de plusieurs séquences à faible abondance nucléotidique, qui diffèrent d'une séquence à forte abondance nucléotidique, par la présence d'un ou de plusieurs changements de base simple et par des insertions ou délétions. Il est possible d'enlever la séquence de type sauvage à forte abondance nucléotidique, de manière sélective, à l'aide d'une conversion analogique très précise par réaction en chaîne de la polymérase, laquelle est facilitée par des conditions tampons optimales, afin de créer un site d'endonuclease de restriction, dans le gène de type sauvage à forte abondance nucléotidique, mais pas dans le gène mutant à faible abondance nucléotidique. Ce processus permet la digestion de l'ADN à forte abondance nucléotidique. Ultérieurement, on amplifie et on détecte l'ADN mutant à faible abondance nucléotidique au moyen d'un dosage de réaction de détection par la ligase. L'invention concerne également une trousse permettant l'exécution de cette procédure.</p>		

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<p>(21) International Application Number: PCT/US00/07133</p> <p>(22) International Filing Date: 17 March 2000 (17.03.00)</p> <p>(30) Priority Data: 60/125,251 19 March 1999 (19.03.99) US</p> <p>(71) Applicants: CORNELL RESEARCH FOUNDATION, INC. [US/US]; 20 Thornwood Drive, Suite 105, Ithaca, NY 14850 (US). LOUISIANA STATE UNIVERSITY [US/US]; 203 David Boyd Hall, 146A, Baton Rouge, LA 70803 (US). PURDUE RESEARCH FOUNDATION [US/US]; 1063 Hovde Hall, Room 300, West Lafayette, IN 47907-1063 (US).</p> <p>(72) Inventors: BARANY, Francis; Apartment 12C, 450 E. 63rd Street, New York, NY 10021 (US). DAY, Joseph, P.; 106 Arcadia Drive, Pacifica, CA 94404 (US). HAMMER, Robert, P.; 4967 Tulane Drive, Baton Rouge, LA 70808 (US). BERGSTROM, Donald, E.; 3416 Hamilton Street, West Lafayette, IN 47906 (US).</p> <p>(74) Agents: GOLDMAN, Michael, L. et al.; Nixon Peabody LLP, Clinton Square, P.O. Box 1051, Rochester, NY 14603 (US).</p>		<p>(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>Without international search report and to be republished upon receipt of that report.</i></p>
<p>(54) Title: COUPLED POLYMERASE CHAIN REACTION-RESTRICTION ENDONUCLEASE DIGESTION-LIGASE DETECTION REACTION PROCESS</p>		
<p>(57) Abstract</p> <p>The present invention provides a method for identifying one or more low abundance sequences differing by one or more single-base changes, insertions, or deletions, from a high abundance sequence in a plurality of target nucleotide sequences. The high abundance wild-type sequence is selectively removed using high fidelity polymerase chain reaction analog conversion, facilitated by optimal buffer conditions, to create a restriction endonuclease site in the high abundance wild-type gene, but not in the low abundance mutant gene. This allows for digestion of the high abundance DNA. Subsequently the low abundant mutant DNA is amplified and detected by the ligase detection reaction assay. The present invention also relates to a kit for carrying out this procedure.</p>		

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Description

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**COUPLED POLYMERASE CHAIN REACTION-RESTRICTION
ENDONUCLEASE DIGESTION-LIGASE DETECTION REACTION
PROCESS**

This application claims the benefit of U.S. Provisional Patent Application Serial No. 60/125,251 filed March 19, 1999.

This invention was developed with government funding under National Institutes of Health Grant Nos. GM-41337-06, GM-43552-05, GM-42722-07, and GM-51628-02. The U.S. Government may have certain rights.

FIELD OF THE INVENTION

The present invention is directed to a process involving coupled polymerase chain reaction ("PCR"), restriction endonuclease digestion ("RE"), and ligase detection reaction ("LDR").

BACKGROUND OF THE INVENTION

Cancer Detection

As the second leading cause of death in this country, almost 600,000 people will die from cancer per year making cancer one of the most alarming of all medical diagnosis. Lifetime risks for developing invasive cancers in men and women are 50 percent and 33 percent, respectively. Expectations are that more than 1.2 million new cases of cancer will be diagnosed in the United States in 1995. Healthcare expenses for cancer in 1994 were approximately \$104 billion. However, the full impact of cancer on families and society is not measured only by the amount of money spent on its diagnosis and treatment. A significant number of people are stricken with cancer in their most productive years. Cancers accounted for 18 percent of premature deaths in 1985 and in 1991 more than 9,200 women in the U.S. died from breast cancer before the age of 55.

Currently, diagnosis of cancer is based on histological evaluation of tumor tissue by a pathologist. After a cancer is diagnosed, treatment is determined primarily by the extent or stage of the tumor. Tumor stage is defined by clinical, radiological, and laboratory methods. Standardized classification

5 systems for the staging of tumors have been developed to clearly convey clinical
information about cancer patients. Staging provides important prognostic
10 information and forms the basis of clinical studies which allow the testing of new
treatment strategies. A staging system was developed (TNM staging system),
5 which classifies tumors according to the size of the primary tumor, the number of
regional lymph nodes in which cancer is found, and the presence or absence of
15 metastases to other parts of the body. Smaller cancers with no affected lymph
nodes and no distant metastases are considered early stage cancers, which are
often amenable to cure through surgical resection. A common measure of
20 prognosis is the 5-year survival rate, the proportion of patients alive five years
after the diagnosis of a cancer at a given stage. While 5-year survival rates for
many cancers have improved over the last few decades, the fact that some early
stage cancers recur within five years or later has led researchers to explore other
25 additional prognostic markers including histological grade, cytometry results,
15 hormone receptor status, and many other tumor markers. Most recently,
investigators have explored the use of molecular alterations in cancers as
prognostic indicators.

30 Genetic alterations found in cancers, such as point mutations and
small deletions can act as markers of malignant cells.

20 Detection of Minority Nucleic Acid Sequences

35 A number of procedures have been disclosed to detect cancer using
PCR. Sidransky, et al., "Identification of *ras* Oncogene Mutations in the Stool of
Patients with Curable Colorectal Tumors," Science 256: 102-05 (1992) detects
40 25 colon cancer by identification of K-*ras* mutations. This involves a PCR
amplification of total DNA, cloning into a phage vector, plating out the phage,
repeated probing with individual oligonucleotides specific to several different K-
45 *ras* mutations, and counting the percentage of positive plaques on a given plate.
This is a technically difficult procedure which takes three days to complete,
30 whereby the ratio of mutant to wild-type DNA in the stool sample is determined.
Brennan, et al., "Molecular Assessment of Histopathological Staging in
50 Squamous-Cell Carcinoma of the Head and Neck," N. Engl. J. Med. 332(7): 429-
35 (1995), finds p53 mutations by sequencing. This specific mutation is then

5 probed for in margin tissue using PCR amplification of total DNA, cloning into a
phage vector, plating out the phage, probing with an individual oligonucleotide
10 specific to the mutation found by sequencing, and counting the percentage of
positive plaques on a given plate. Berthelemy, et al., "Brief Communications--
5 Identification of K-*ras* Mutations in Pancreatic Juice in the Early Diagnosis of
Pancreatic Cancer," Ann. Int. Med. 123(3): 188-91 (1995) uses a PCR/restriction
15 enzyme process to detect K-*ras* mutations in pancreatic secretions. This technique
is deficient, however, in that mutations are not quantified. Similarly, Tada, et al.,
"Detection of *ras* Gene Mutations in Pancreatic Juice and Peripheral Blood of
20 Patients with Pancreatic Adenocarcinoma," Cancer Res. 53: 2472-74 (1993) and
Tada, et al., "Clinical Application of *ras* Gene Mutation for Diagnosis of
Pancreatic Adenocarcinoma," Gastroent. 100: 233-38 (1991) subject such samples
25 to allele-specific PCR to detect pancreatic cancer. This has the disadvantages of
providing false positives due to polymerase extension off normal template,
15 requiring electrophoretic separation of products to distinguish from primer dimers,
being unable to multiplex closely-clustered sites due to interference of
30 overlapping primers, being unable to detect single base or small insertions and
deletions in small repeat sequences, and not being practically suitable for
quantification of mutant DNA in a high background of normal DNA. Hayashi, et
20 al., "Genetic Detection Identifies Occult Lymph Node Metastases Undetectable by
the Histopathological Method," Cancer Res. 54: 3853-56 (1994) uses an allele-
35 specific PCR technique to find K-*ras* or p53 mutations to identify occult lymph
node metastases in colon cancers. A sensitivity of one tumor cell in one thousand
of normal cells is claimed; however, obtaining quantitative values requires
40 laborious cloning, plating, and probing procedures. In Mitsudomi, et al.,
"Mutations of *ras* Genes Distinguish a Subset of Non-small-cell Lung Cancer Cell
Lines from Small-cell Lung Cancer Cell Lines," Oncogene 6: 1353-62 (1993),
45 human lung cancer cell lines are screened for point mutations of the K-, H-, and
N-*ras* genes using restriction fragment length polymorphisms created through
30 mismatched primers during PCR amplification of genomic DNA. The
disadvantages of such primer-mediated RFLP include the requirement of
50 electrophoretic separation to distinguish mutant from normal DNA, limited
applicability to sites that may be converted into a restriction site, the requirement

5 for additional analysis to determine the nature of the mutation, and the difficulty
in quantifying mutant DNA in a high background of normal DNA. Further, these
10 procedures tend to be laborious and inaccurate.

Coupled PCR/ligation processes have been used for detection of
5 minority nucleotide sequences in the presence of majority nucleotide sequences.
A PCR/LDR process is used in Frenkel, "Specific, Sensitive, and Rapid Assay for
15 Human Immunodeficiency Virus Type 1 *pol* Mutations Associated with
Resistance to Zidovudine and Didanosine," J. Clin. Microbiol. 33(2): 342-47
(1995) to detect HIV mutants. This assay, however, cannot be used for multiplex
20 detection. See also Abravaya, et al., "Detection of Point Mutations With a
Modified Ligase Chain (Gap-LCR)," Nucl. Acids Res. 23(4): 675-82 (1995) and
Balles, et al., "Facilitated Isolation of Rare Recombinants by Ligase Chain
Reaction: Selection for Intragenic Crossover Events in the *Drosophila optomotor-*
25 *blind* Gene," Molec. Gen. Genet. 245: 734-40 (1994).

Colorectal lesions have been detected by a process involving PCR
15 amplification followed by an oligonucleotide ligation assay. See Jen, et al.,
"Molecular Determinants of Dysplasia in Colorectal Lesions," Cancer Res. 54:
30 5523-26 (1994) and Redston, et al., "Common Occurrence of *APC* and *K-ras*
Gene Mutations in the Spectrum of Colitis-Associated Neoplasias," Gastroenter.
20 108: 383-92 (1995). This process was developed as an advance over Powell, et
al., "Molecular Diagnosis of Familial Adenomatous Polyposis," N. Engl. J. Med.
35 329(27): 1982-87 (1993). These techniques tend to be limited and difficult to
carry out.

Other procedures have been developed to detect minority
40 nucleotide sequences. Lu, et al., "Quantitative Aspects of the Mutant Analysis by
25 PCR and Restriction Enzyme Cleavage (MAPREC)" PCR Methods and Appl. 3:
176-80 (1993) detects virus revertants by PCR and restriction enzyme cleavage.
45 The disadvantages of MAPREC include the requirement for electrophoretic
separation to distinguish mutant from normal DNA, limited applicability to sites
30 that may be converted into a restriction site, the requirement for additional
analysis to determine the nature of the mutation, and difficulty in quantifying
50 mutant DNA in a high background of normal DNA. In Kuppuswamy, et al.,
"Single Nucleotide Primer Extension to Detect Genetic Diseases: Experimental
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Application to Hemophilia G (Factor IX) and Cystic Fibrosis Genes," Proc. Natl. Acad. Sci. USA 88: 1143-47 (1991), a PCR process is carried out using two reaction mixtures for each fragment to be amplified with one mixture containing a primer and a labeled nucleotide corresponding to the normal coding sequence, while the other mixture contains a primer and a labeled nucleotide corresponding to the mutant sequence. The disadvantages of such mini sequencing (i.e. SNuPe) are that the mutations must be known, it is not possible to multiplex closely clustered sites due to interference of overlapping primers, it is not possible to detect single base or small insertions and deletions in small repeat sequences, and four separate reactions are required. A mutagenically separated PCR process is disclosed in Rust, et al., "Mutagenically Separated PCR (MS-PCR): a Highly Specific One Step Procedure for easy Mutation Detection" Nucl. Acids Res. 21(16): 3623-29 (1993) to distinguish normal and mutant alleles, using different length allele-specific primers. The disadvantages of MS-PCR include possibly providing false positives due to polymerase extension off normal template, requiring electrophoretic separation of products to distinguish from primer dimers, the inability to multiplex closely-clustered sites due to interference of overlapping primers, the inability to detect single base or small insertions and deletions in small repeat sequences, and not being ideally suited for quantification of mutant DNA in high background of normal DNA. In Suzuki, et al., "Detection of *ras* Gene Mutations in Human Lung Cancers by Single-Strand Conformation Polymorphism Analysis of Polymerase Chain Reaction Products," Oncogene 5: 1037-43 (1990), mutations are detected in a process having a PCR phase followed by a phase involving single strand conformation polymorphism ("SSCP") of the amplified DNA fragments. The disadvantages of SSCP include the requirement for electrophoretic separation to distinguish mutant conformer from normal conformer, the failure to detect 30% of possible mutations, the requirement for additional analysis to determine the nature of the mutation, and the inability to distinguish mutant from silent polymorphisms.

Nucleotide Conversion Fidelity

Many of the approaches to detecting the presence of a given sequence or sequences in a polynucleotide sample involve amplification of the minority

sequence(s) by polymerase chain reaction (PCR). U.S. Patent No. 4,683,202 to Mullis, et al., and R.K. Saiki, et al., Science 230:1350 (1985). In this method, primers complementary to opposite end portions of the selected sequence(s) are used to promote, in conjunction with thermal cycling, successive rounds of primer-initiated replication. The amplified sequence(s) may be readily identified by a variety of techniques. This approach is particularly useful for detecting the presence of low-copy sequences in a polynucleotide-containing sample, e.g., for detecting pathogen sequences in a body-fluid sample. However, a nonselective PCR strategy will amplify both mutant and wild-type alleles with approximately equal efficiency, resulting in low abundance mutant alleles comprising only a small fraction of the final product. If the mutant sequence comprises less than 25% of the amplified product, it is unlikely that DNA sequencing will be able to detect the presence of such an allele. Although it is possible to accurately quantify low abundance mutations by first separating the PCR products by cloning and subsequently probing the clones with allele-specific oligonucleotides (ASOs) (Saiki et al., "Analysis of Enzymatically Amplified Beta-Globin and HLA-DQ Alpha DNA with Allele-Specific Oligonucleotide Probes," Nature, 324(6093):163-6 (1986); Sidransky et al., "Identification of Ras Oncogene Mutations in the Stool of Patients with Curable Colorectal Tumors," Science, 256:102-5 (1992); and Brennan et al., "Molecular Assessment of Histopathological Staging in Squamous-Cell Carcinoma of the Head And Neck," N. Engl. J. Med., 332(7):429-35 (1995)), this approach is time consuming. In contrast, allele-specific PCR methods can rapidly and preferentially amplify mutant alleles. For example, multiple mismatch primers have been used to detect H-ras mutations at a sensitivity of 1 mutant in 10⁵ wild-type alleles (Cha et al., "Mismatch Amplification Mutation Assay (MAMA): Application to the C-H-Ras Gene," PCR Methods Appl., 2(1):14-20 (1992)) and claims as high as 1 mutant in 10⁶ wild-type alleles have been reported (Iliassos et al., "Detection of Minority Point Mutations by Modified PCR Technique: A New Approach for a Sensitive Diagnosis of Tumor-Progression Markers," Nucleic Acids Res., 17:8093-9 (1989); and Chen et al., "A Nonradioactive, Allele-Specific Polymerase Chain Reaction for Reproducible Detection of Rare Mutations in Large Amounts of Genomic DNA: Application to Human K-Ras," Anal. Biochem., 244:191-4

(1997)). However, careful evaluation suggests these successes are limited to allele-specific primers discriminating through 3' purine-purine mismatches. For the more common transition mutations, the discriminating mismatch on the 3' primer end (*i.e.*, G:T or C:A mismatch) will be removed in a small fraction of products by polymerase error during extension from the opposite primer on wild-type DNA. Thereafter, these error products are efficiently amplified and generate false-positive signal. One strategy to eliminate this polymerase error problem is to deplete wild-type DNA early in PCR.

Several investigators have explored selective removal of wild-type DNA by restriction endonuclease (RE) digestion in order to enrich for low abundance mutant sequences. These RFLP methods detect approximately 1 mutant in 10⁶ wild-type or better by combining the sensitivity of polymerase with the specificity of restriction endonucleases. One approach has used digestion of genomic DNA followed by PCR amplification of the uncut fragments (RFLP-PCR) to detect very low-level mutations within restriction sites in the *H-ras* and *p53* genes (Sandy et al., "Genotypic Analysis of Mutations in Taq I Restriction Recognition Sites by Restriction Fragment Length Polymorphism/Polymerase Chain Reaction," *Proc. Natl. Acad. Sci. USA*, 89:890-4 (1992); and Pourzand et al., "Genotypic Mutation Analysis by RFLP/PCR," *Mutat. Res.*, 88(1):113-21 (1993)). Similar results have been obtained by digestion following PCR and subsequent amplification of the uncleaved DNA now enriched for mutant alleles (PCR-RFLP) (Kumar et al., "Oncogene Detection at the Single Cell Level," *Oncogene*, 3(6):647-51 (1988); Kumar et al., "Designed Diagnostic Restriction Fragment Length Polymorphisms for The Detection of Point Mutations in Ras Oncogenes," *Oncogene Res.*, 4(3):235-41 (1989); and Jacobson et al., "A Highly Sensitive Assay for Mutant Ras Genes and its Application to the Study of Presentation and Relapse Genotypes in Acute Leukemia," *Oncogene*, 9(2):553-63 (1994)). Although sensitive and rapid, RFLP detection methods are limited by the requirement that the location of the mutations must coincide with restriction endonuclease recognition sequences. To circumvent this limitation, primers that introduce a new restriction site have been employed in "primer-mediated" RFLP (Jacobson et al., "Rapid, Nonradioactive Screening for Activating Ras Oncogene Mutations Using PCR-Primer Introduced Restriction Analysis (PCR-PIRA)," PCR

5 Methods Appl., 1(4):299 (1992); Chen et al., "A Method to Detect Ras Point
Mutations in Small Subpopulations of Cells," Anal. Biochem., 195(1):51-6
10 (1991); Di Giuseppe et al., "Detection of K-Ras Mutations in Mucinous
Pancreatic Duct Hyperplasia from a Patient with a Family History of Pancreatic
5 Carcinoma," Am. J. Pathol., 144(5):889-95 (1994); Kahn et al., "Rapid and
Sensitive Nonradioactive Detection of Mutant K-Ras Genes Via 'Enriched' PCR
15 Amplification," Oncogene, 6:1079-83 (1991); Levi et al., "Multiple K-Ras Codon
12 Mutations in Cholangiocarcinomas Demonstrated with a Sensitive Polymerase
Chain Reaction Technique," Cancer Research, 51(July):3497-502 (1991); and
20 Mitsudomi et al., "Mutations of Ras Genes Distinguish a Subset of Non-Small-
Cell Lung Cancer Cell Lines from Small-Cell Lung Cancer Cell Lines,"
Oncogene, 6(8):1353-62 (1991)). However, subsequent investigators have
demonstrated that errors are produced at the very next base by polymerase
25 extension from primers having 3' natural base mismatches (Hattori et al.,
15 "Mismatch PCR RFLP Detection of DRD2 Ser311Cys Polymorphism and
Schizophrenia," Biochem. Biophys. Res. Commun., 202(2):757-63 (1994); O'Dell
et al., "PCR Induction of a *TaqI* Restriction Site at Any CpG Dinucleotide Using
30 Two Mismatched Primers (CpG-PCR)," Genome Res., 6(6):558-68 (1996); and
Hodanova et al., "Incorrect Assignment of N370S Mutation Status by Mismatched
20 PCR/RFLP Method in Two Gaucher Patients," J. Inherit. Metab. Dis., 20(4):611-2
(1997)). Such templates fail to cleave during restriction digestion and amplify as
35 false-positives that are indistinguishable from true positive products extended
from mutant templates.

40 Use of nucleotide analogs may reduce errors resulting from
25 polymerase extension and improve base conversion fidelity. Nucleotide analogs
that are designed to base-pair with more than one of the four natural bases are
termed "convertides." Base incorporation opposite different convertides has been
45 tested (Hoops et al., "Template Directed Incorporation of Nucleotide Mixtures
Using Azole-Nucleobase Analogs," Nucleic Acids Res., 25(24):4866-71 (1997)).
30 For each analog, PCR products were generated using *Taq* polymerase and primers
containing an internal nucleotide analog. The products generated showed a
50 characteristic distribution of the four bases incorporated opposite the analogs. Of
significance, these products retained the original sequence at all natural base

positions. Convertides readily form degenerate amplification products by virtue of their ability to assume different hydrogen bonding patterns through tautomeric shift (Brown et al., "Synthesis and Duplex Stability of Oligonucleotides Containing Adenine-Guanine Analogues," Carbohydrate Research, 216:129-39 (1991)), bond rotation (Bergstrom et al., Nucleosides and Nucleotides, 15(1-3):59-68 (1996)), or base stacking (Bergstrom et al., Journal of the American Chemical Society, 117:1201-9 (1995) and Zhang et al., "Exploratory Studies on Azole Carboxamides as Nucleobase Analogs: Thermal Denaturation Studies on Oligodeoxyribonucleotide Duplexes Containing Pyrrole-3-Carboxamide," Nucleic Acids Res., 26:2208-15 (1998)). Thus, PCR primers containing convertides may be used to facilitate base conversion. In principle, using the 6H,8H-3,4-dihydropyrimido[4,5-c][1,2]oxazine-7-one analog Q₆, which is known to exhibit both the C-like and T-like tautomeric forms at the 3' end of the primer (Brown et al., "Synthesis and Duplex Stability of Oligonucleotides Containing Adenine-Guanine Analogues," Carbohydrate Research, 216:129-39 (1991)), a C-G base-pair may be converted to a T-A base pair (Figure 1). Due to the better geometry, DNA polymerases may "read," or extend better, from a Q₆-G pair than a T-G mismatch (wobble base pair). Similarly, DNA polymerases may "write," or incorporate both G and A bases opposite Q₆ (Hill et al., "Polymerase Recognition of Synthetic Oligodeoxyribonucleotides Incorporating Degenerate Pyrimidine and Purine Bases," Proc. Natl. Acad. Sci. USA, 95(8):4258-63 (1998)), whereas A is always inserted opposite a T base. Thus, the Q₆ analog primer serves as an intermediary, providing a "preconversion" step before a natural base primer is added to selectively amplify the desired product from the degenerate pool. While nucleotide analogs have great potential, they have not been tested in high sensitivity assays. There is a need for a method that optimizes the fidelity of the analog conversion in the PCR process.

Optimization of PCR/RE/LDR

As discussed above, PCR used with a high fidelity conversion process would provide a valuable method for the amplification of mutant gene sequences. By designing primers with one or more mismatches, mutant DNA template can be efficiently extended, while poor extension is achieved on wild-

type DNA template. However, once these primers extend with or without a mismatch, the products thereafter are perfect matches for the primer in subsequent PCR cycles. Thus, false positive signals are amplified in subsequent cycles. Moreover, PCR error can generate a base change in the template, which perfectly matches the primer. AS-PCR can detect pyrimidine↔purine transversions at sensitivities of 1 in 10^5 (Newton et al., "Analysis of Any Point Mutation in DNA. The Amplification Refractory Mutation System (ARMS)," Nucleic Acids Res., 17(7):2503-16 (1989); and Tada et al., "Detection of Ras Gene Mutations in Pancreatic Juice and Peripheral Blood of Patients with Pancreatic Adenocarcinoma," Cancer Res., 53(11):2472-4 (1993)). Nevertheless, the majority of cancer-associated mutations are C↔T and A↔G transitions, as, for example, are over 80% of p53 point mutations (de Fromental et al., Genes Chromosomes Cancer, 4(1):1-15 (1992)). A DNA diagnostic method is needed to accurately quantify this type of low abundance mutation.

The ligation detection reaction (LDR) in conjunction with PCR has been used to quantify small amounts of PCR extension product. LDR uses two adjacent primers and a thermostable ligase to distinguish all four bases potentially found at any position in a DNA sequence (Barany, F., "Genetic Disease Detection and DNA Amplification Using Cloned Thermostable Ligase," Proc. Natl. Acad. Sci. USA, 88:189-93 (1991); Barany, F., "The Ligase Chain Reaction in a PCR World," PCR Methods Appl., 1:5-16 (1991); Day et al., "Detection of Steroid 21-Hydroxylase Alleles Using Gene-Specific PCR and a Multiplexed Ligation Detection Reaction," Genomics, 29:152-62 (1995); and Khanna et al., Oncogene, 18:27-38 (1999)). Thermostable ligase demonstrates the highest fidelity when the discriminating base is located at the 3' end of the upstream primer (Luo et al., "Improving the Fidelity of *Thermus Thermophilus* DNA Ligase," Nucleic Acids Res., 24(15):3071-8 (1996)). PCR/LDR (PCR of a sequence from genomic DNA followed by LDR) can detect mutations with a sensitivity of approximately 1 mutant allele in 4,000 normal alleles (Khanna et al., Oncogene, 18:27-38 (1999)). Sensitivity of approximately 1 in 10^6 has been achieved by combining PCR with restriction endonuclease digestion of wild-type DNA (Sandy et al., Proc. Natl. Acad. Sci. USA, 89:890-4 (1992)); and Pourzand et al., "Genotypic Mutation Analysis by RFLP/PCR," Mutat. Res., 288(1):113-21 (1993)). Mutations

5 occurring within the restriction site prevent cleavage of the mutant allele, while
wild-type alleles bearing canonical restriction site sequence are depleted. As a
10 result, subsequent PCR cycles preferentially amplify mutant DNA. If a mutation
site is not within an endonuclease recognition site present in wild-type DNA, a
5 restriction site must be introduced. This is typically done by PCR using a primer
or primers with mismatched bases. Mutations cannot be detected in any portion of
15 the restriction site spanned by the primers, since those bases are introduced
directly through the primers. In a random DNA sequence, over 20% of bases are
contained within a preexisting four-base restriction site and 60% of bases are
20 within a four-base subsequence that can be converted into a restriction site by a
single base change. In these small sites, 3' terminal base mismatch primers must
frequently be used. While conceptually straightforward, 3' mismatch extension
has proven to be difficult (Newton et al., "Analysis of Any Point Mutation in
25 DNA. The Amplification Refractory Mutation System (ARMS)," Nucleic Acids
15 Res., 17(7):2503-16 (1989); Kwok et al., "Effects of Primer-Template
Mismatches on the Polymerase Chain Reaction: Human Immunodeficiency Virus
Type 1 Model Studies," Nucleic Acids Res., 18(4):999-1005 (1990); O'Dell et al.,
30 Genome Res., 6(6):558-68 (1996); and Day et al., Nucleic Acids Res., (1999)).
The introduction of interrupted palindromic restriction sites has been more
20 successful using internal mismatch primers spanning one half-site through the
intervening bases up to the other half-site (Kumar et al., "Oncogene Detection at
35 the Single Cell Level," Oncogene, 3(6):647-51 (1988); and Anderson et al.,
"Prevalence of RAS Oncogene Mutation in Head and Neck Carcinomas," J.
40 Otolaryngol., 21(5):321-6 (1992)). Several perfectly matched bases stabilize the
25 3' end of the mismatch primer. However, this approach may be used only if the
second half-site is present naturally in wild-type DNA. Mutations in the second
half-site prevent digestion. Only mutations occurring at bases within the
45 recognition sequence are detectable by RFLP methods. Mutations occurring at
bases outside a preexisting restriction site in wild-type DNA may be detected by
30 introducing a new restriction site containing that base.

50 Restriction endonucleases recognizing interrupted palindromes are
less abundant than endonucleases recognizing contiguous four- and six-base sites.

Multiple base changes would often be required to introduce an interrupted palindrome restriction site to identify mutations at any base.

More recently, methods of identifying known target sequences by probe ligation methods have been reported. U.S. Patent No. 4,883,750 to N.M. Whiteley, et al.; D.Y. Wu, et al., Genomics 4:560 (1989), U. Landegren, et al., Science 241:1077 (1988), and E. Winn-Deen, et al., Clin. Chem. 37:1522 (1991). In one approach, known as oligonucleotide ligation assay ("OLA"), two probes or probe elements, that span a target region of interest, are hybridized to the target region. Where the probe elements base pair with adjacent target bases, the confronting ends of the probe elements can be joined by ligation, e.g., by treatment with ligase. The ligated probe element is then assayed, evidencing the presence of the target sequence.

In a modification of this approach, the ligated probe elements act as a template for a pair of complementary probe elements. With continued cycles of denaturation, hybridization, and ligation in the presence of pairs of probe elements, the target sequence is amplified linearly, allowing very small amounts of target sequence to be detected and/or amplified. This approach is referred to as ligase detection reaction. When two complementary pairs of probe elements are utilized, the process is referred to as the ligase chain reaction, which achieves exponential amplification of target sequences. F. Barany, "Genetic Disease Detection and DNA Amplification Using Cloned Thermostable Ligase," Proc. Nat'l Acad. Sci. USA, 88:189-93 (1991) and F. Barany, "The Ligase Chain Reaction (LCR) in a PCR World," PCR Methods and Applications, 1:5-16 (1991); Barany, U.S. Patent Application filed March 19, 1999, SN 60/125,251. Techniques, such as PCR/LDR, that rely on mutant enrichment require optimization of reaction conditions in order to minimize random PCR errors. These errors would be indistinguishable from mutations originally present in clinical samples. One source of error-minimization may be found in optimization of the buffer conditions for PCR. Standard PCR buffers contain Tris, however the pK_a of Tris is strongly dependent on temperature. A PCR reaction containing Tris pH 8.3 (measured at 23°C) is approximately pH 7 near 65°C (the extension temperature), and drops to approximately pH 6 near 95°C (the template melting temperature). PCR error can result from template degradation and polymerase

misincorporation. Template degradation occurs during periods of high temperature and low pH in each PCR cycle and limits product size in "long" PCR (Barnes, "PCR Amplification of up to 35-Kb DNA with High Fidelity and High Yield from Lambda Bacteriophage Templates," Proc. Natl. Acad. Sci. USA, 91(6):2216-20 (1994); Cheng et al., "Effective Amplification of Long Targets from Cloned Inserts and Human Genomic DNA," Proc. Natl. Acad. Sci. USA, 91(12):5695-9 (1994); and Sang et al., "Generation of Site-Directed Mutagenesis by Extralong, High-Fidelity Polymerase Chain Reaction," Anal. Biochem., 233(1):142-4 (1996)). Raising the buffer pH in long PCR (using Tris 9.1) reduces the amount of template cleavage and increases PCR efficiency (Barnes, "PCR Amplification of up to 35-Kb DNA with High Fidelity and High Yield from Lambda Bacteriophage Templates," Proc. Natl. Acad. Sci. USA, 91(6):2216-20 (1994)). Although the efficiency of long PCR increases with higher pH, the level of mutations within these PCR products may also increase since high pH decreases the fidelity of *Taq* and *Pfu* polymerases (Eckert et al., "High Fidelity DNA Synthesis by the *Thermus Aquaticus* DNA Polymerase," Nucleic Acids Res., 18(13):3739-44 (1990); Eckert et al., "DNA Polymerase Fidelity and the Polymerase Chain Reaction," PCR Methods Appl., 1(1):17-24 (1991); and Cline et al., "PCR Fidelity of *Pfu* DNA Polymerase and Other Thermostable DNA Polymerases," Nucleic Acids Res., 24(18):3546-51 (1996)). Use of alternative PCR buffers with lower $|\Delta pK_a|$ can improve polymerase fidelity and still reduce template damage by maintaining more neutral pH over a wider temperature range (Eckert et al., "DNA Polymerase Fidelity and the Polymerase Chain Reaction," PCR Methods Appl., 1(1):17-24 (1991); and Brail et al., "Improved Polymerase Fidelity in PCR-SSCPA," Mutat. Res., 303(4):171-5 (1993)). The addition of glycerol or formamide may reduce mutations arising from template damage during PCR cycling and may help avoid misextension from mispaired primers (Bottema et al., "PCR Amplification of Specific Alleles: Rapid Detection Of Known Mutations and Polymorphisms," Mutat. Res., 288(1):93-102 (1993); and Cha et al., "Mismatch Amplification Mutation Assay (MAMA): Application to the C-H-Ras Gene," PCR Methods Appl., 2(1):14-20 (1992)).

Thus, there is a need to improve buffer reaction conditions currently used in PCR, in order to minimize the opportunity for mismatches

caused by PCR error. Increased analog conversion fidelity, alone, will not solve the need for an improved method of mutant DNA detection. In addition, there is a need to optimize PCR reaction conditions to decrease random PCR error, and finally, a method is needed that provides sensitive detection for the PCR extension products.

SUMMARY OF THE INVENTION

The present invention relates to a method for identifying one or more low abundance sequences differing by one or more single-base changes, insertions, or deletions, from a high abundance sequence, in a plurality of target nucleotide sequences. This method involves a first polymerase chain reaction phase, a second polymerase chain reaction phase, and a restriction endonuclease digest reaction phase, followed by a third polymerase chain reaction phase and a ligase detection reaction phase.

The starting sample of the present invention is a sample potentially containing one or more low abundance target nucleotide sequences with at least one sequence difference each from the high abundant target sequences present in the sample.

In the first polymerase chain reaction phase, a primary oligonucleotide primer set is provided. The primary oligonucleotide primer set has a first oligonucleotide primer containing a target-specific portion, and a second oligonucleotide primer containing a target-specific portion. The primary oligonucleotide primers are suitable for hybridization on complementary strands of a corresponding high and low abundance target nucleotide sequences to permit formation of a polymerase chain reaction product. However, the primers each have a mismatch which interferes with formation of such a polymerase chain reaction product when hybridized to any other nucleotide sequence present in the sample. The primary oligonucleotide primers, the sample, and a polymerase are blended to form a primary polymerase chain reaction mixture.

The primary polymerase chain reaction mixture is subjected to two or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment. The denaturing treatment

5 separates hybridized nucleic acid sequences. The hybridization treatment causes
the target-specific portions of the primary oligonucleotide primers to hybridize to
10 the target nucleotide sequences. The extension treatment causes the hybridized
primary oligonucleotide primers to be extended to form primary extension
5 products complementary to the target nucleotide sequence to which the primary
oligonucleotide primer is hybridized.

15 Next, there is a second polymerase chain reaction phase. This
phase involves providing a secondary oligonucleotide primer set having a first
oligonucleotide primer with a target-specific portion and a 5' upstream secondary
20 primer-specific portion and a second oligonucleotide primer with a target-specific
portion and a 5' upstream secondary primer-specific portion. The secondary
oligonucleotide primers in a particular set are suitable for hybridization on
complementary strands of the primary extension products to permit formation of a
25 secondary polymerase chain reaction product which contains or creates a
restriction endonuclease recognition site when amplifying the high abundance
15 target, but does not contain or create a restriction endonuclease recognition site
when amplifying the one or more low abundance targets. The primary extension
30 products, the secondary oligonucleotide primers, and the polymerase are blended
to form a secondary polymerase chain reaction mixture.

20 The secondary polymerase chain reaction mixture is subjected to
two or more polymerase chain reaction cycles comprising a denaturation
35 treatment, a hybridization treatment, and an extension treatment. The
denaturation treatment involves separation of hybridized nucleic acid sequences.
In the hybridization treatment, the secondary oligonucleotide primers hybridize to
40 the primary extension products. The extension treatment causes the hybridized
25 primary extension products to form secondary extension products complementary
to the primary extension products. The high abundance secondary extension
45 products contain a restriction site but the low abundance secondary extension
products do not.

30 The next phase involves blending a restriction endonuclease with
the secondary extension products to form an endonuclease digestion reaction
50 mixture. The restriction endonuclease is one that cleaves, with specificity, the
restriction endonuclease recognition site within or created when amplifying the

5 high abundance target, but not the low abundance target in the secondary extension products. The restriction endonuclease digestion selectively destroys
10 the high abundance secondary extension products.

10 Next, there is a third polymerase chain reaction phase. This
5 involves providing a tertiary oligonucleotide primer set having a first tertiary primer containing the same sequence as the 5' upstream portion of the first
15 oligonucleotide primer of the secondary oligonucleotide primer set and a second tertiary primer containing the same sequence as the 5' upstream portion of a
20 second oligonucleotide primer of the secondary oligonucleotide primer set. The set of tertiary oligonucleotide primers may be used to amplify all of the secondary
extension products. The secondary extension products are blended with the tertiary oligonucleotide primer set, and a polymerase to form a tertiary polymerase
chain reaction mixture.

25 The tertiary polymerase chain reaction mixture is subjected to two
15 or more polymerase chain reaction cycles comprising a denaturation treatment, a hybridization treatment, and an extension treatment. The denaturation treatment
causes the hybridized nucleic acid sequences to be separated, while the
30 hybridization treatment involves hybridization of the tertiary oligonucleotide primers to hybridize to the secondary extension products. During the extension
20 treatment, the hybridized tertiary oligonucleotide primers are extended to form tertiary extension products complementary to the uncleaved secondary extension
35 products.

Next, the tertiary extension products are subjected to a ligase
40 detection reaction. This involves providing a plurality of oligonucleotide probe sets, each set having a first oligonucleotide probe, having a tertiary extension
25 product-specific portion and a detectable reporter label, and a second oligonucleotide probe, having a tertiary extension product-specific portion. The
oligonucleotide probes in a particular LDR probe set are suitable for ligation when
45 hybridized adjacent to one another on a complementary tertiary extension product-specific portion. However, there is a mismatch which interferes with such ligation
30 when hybridized to any other nucleotide sequence present in the sample.

50 The tertiary extension products, the plurality of oligonucleotide probe sets, and a ligase are blended to form a ligase detection reaction mixture.

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The ligase detection reaction mixture is subjected to one or more ligase detection reaction cycles having a denaturation treatment, and a hybridization treatment. The denaturation treatment involves separation of hybridized oligonucleotides from the tertiary extension products. In the hybridization treatment, the oligonucleotide probe sets hybridize at adjacent positions in a base-specific manner to their respective tertiary extension products, if present. As a result, adjacent probes ligate to one another to form a ligation product sequence containing the detectable reporter label and the tertiary extension product-specific portions connected together. The oligonucleotide probe sets may hybridize to nucleotide sequences other than their respective complementary tertiary extension products but do not ligate together due to a presence of one or more mismatches and individually separate during the denaturation treatment. Following the ligase detection reaction cycles, the reporter labels of the ligation product sequences are detected which indicates the presence of one or more low abundance target nucleotide sequences in the sample.

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The present invention also relates to a kit for identifying one or more low abundance sequences differing by one or more single-base changes, insertions, or deletions, from a high abundance sequence in a plurality of target nucleotide sequences. This kit provides a primary oligonucleotide primer set, a secondary oligonucleotide primer set, a tertiary oligonucleotide primer set, and a plurality of oligonucleotide probe sets.

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The primary oligonucleotide primer set provided in the kit has (a) a first oligonucleotide primer containing a target-specific portion, and (b) a second oligonucleotide primer containing a target-specific portion. The primary oligonucleotide primers are suitable for hybridization on complementary strands of a corresponding high and low abundance target nucleotide sequences to permit formation of a primary extension product. However, the primers have a mismatch which interferes with formation of such a polymerase chain reaction product when hybridized to any other nucleotide sequence present in the sample.

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The secondary oligonucleotide primer set provided in the kit has (a) a first oligonucleotide primer, containing a target-specific portion and a 5' upstream secondary primer-specific portion, and (b) a second oligonucleotide primer, containing a target-specific portion and a 5' upstream secondary primer-

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specific portion. The secondary oligonucleotide primers are suitable for hybridization on complementary strands of the primary extension products to permit formation of a secondary extension product which contains or creates a restriction endonuclease recognition site when amplifying the high abundance target, but does not contain or create a restriction endonuclease recognition site when amplifying the one or more low abundance targets.

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The tertiary oligonucleotide primer set provided in the kit have (a) a first tertiary primer containing the same sequence as the 5' upstream portion of the first oligonucleotide primer of the secondary oligonucleotide primer set, and (b) a second tertiary primer containing the same sequence as the 5' upstream portion of a second oligonucleotide primer of the secondary oligonucleotide primer set. The set of tertiary oligonucleotide primers may be used to amplify all of the secondary extension products.

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The kit also provides a plurality of oligonucleotide probe sets. Each set has (a) a first oligonucleotide probe, containing a tertiary extension product-specific portion and a detectable reporter label, and (b) a second oligonucleotide probe, containing a tertiary extension product-specific portion. The oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a complementary tertiary extension product-specific portion. However, the probes have a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence present in the sample.

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There is not currently available a biological technique that is sensitive enough to detect and identify one or more low abundance sequences differing by one or more single-base changes, insertions, or deletions, from a high abundance sequence, in a plurality of target nucleotide sequences, without the potential for many errors during the amplification and detection stages. The present invention is directed to overcoming these and other deficiencies in the art, by providing a method which optimizes the conditions for selective amplification of low abundance DNA targets through polymerase chain reaction and restriction endonuclease digestion, and a method of nucleotide detection, that combined, creates a highly sensitive method for the amplification and detection of low

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abundance nucleotides sequences such as mutant genes. This procedure is useful for clinical and research applications.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic drawing of the coupled polymerase chain reaction-restriction endonuclease digestion-ligase detection reaction process of the present invention.

Figure 2 shows the detection of ligation products resulting from the process of Figure 1, using gel electrophoresis.

Figure 3 shows the detection of ligation products resulting from the process of Figure 1 using an addressable array.

Figure 4 shows a conversion facilitated by nucleotide analog preconversion. A C:G base pair in a sequence is targeted for conversion to a T:A basepair. Rather than using a 3' natural base mismatch primer to attempt direct conversion, a nucleotide analog (Q₆) primer is used for preconversion. The Q₆ analog reads the G base well and allows polymerase to efficiently extend from the 3' Q₆ primer. During PCR, the reverse primer anneals to the Q₆ PCR product and is extended by polymerase to synthesize the opposite strand. When polymerase reaches the Q₆ analog in the template, polymerase writes A (or G, not shown) opposite the analog and continues synthesis of the strand. After a few cycles, a pool of products is made with degenerate sequence opposite the analogs. A natural base primer is then added to selectively amplify the products having the desired base change.

Figure 5A-I shows the nucleotide analogs used in PCR primers. In the final deprotected oligonucleotide, the name of the nucleoside containing the base analog shown are as follows: Q₁, 1-(2'-deoxy-β-D-ribofuranosyl)imidazole-4-carboxamide (Figure 5A); Q₂, 1-(2'-deoxy-β-D-ribofuranosyl)-3-nitropyrrole (Figure 5B); Q₃, 2'-deoxyinosine (Figure 5C); Q₄, 6-(2'-deoxy-β-D-ribofuranosyl)-6H,8H-3,4-dihydropyrimido[4,5-c][1,2]oxazine-7-one (Figure 5D); Q₅, 2-amino-7-(2'-deoxy-β-D-ribofuranosyl)-6-methoxyaminopurine (Figure 5E); Q₆, 1-(2'-deoxy-β-D-ribofuranosyl)-4-iodopyrazole (Figure 5F); Q₇, 1-(2'-

deoxy- β -D-ribofuranosyl)pyrrole-3-carboxamide (Figure 5G); **Q**₁₉, 1-(2'-deoxy- β -D-ribofuranosyl)-4-nitropyrrole (Figure 5H). Base analogs (**Q**) are attached to the 1' position of deoxyribofuranose. The nucleoside analogs are attached to the controlled pore glass (CPG) column via a succinyl linker (**R** = linker to CPG).

The oligonucleotide is synthesized from the 5' hydroxyl after removal of the dimethoxytrityl (DMT) protecting group, placing the analog at the 3' end. After cleavage from the CPG column and deprotection, the oligonucleotide is extended by polymerase from the 3' base analog hydroxyl group (**R** = H) (Figure 5I).

Figures 6A-C show the primers used in mismatch extension and 3' nucleotide analog conversion. Complementary (- strand) sequences are shown in reverse orientation (3'-5'), e.g., reverse strand primers (names ending in "R"). In Figure 6A, one of nine different synthetic 50 bp duplex templates is shown melted with primers aligned to complementary sequence. Primer extension was performed using 3' natural base and nucleotide analog primers (p53-248X and p53-248XR). Some extension products were reamplified using truncated zipcode primers p53zip248 and p53zip248R and sequenced using one of the zipcode primers (Ztop or Zbot). In Figure 6B, preconversion was performed on nine different 50 bp synthetic duplex templates using 3' nucleotide analog primers, e.g., p53-248Q₆ and p53-248Q₆R. Conversion, with or without preconversion, was performed using primers containing 3' natural base, e.g. primers p53zip248T and p53zip248TR. These conversion products were reamplified using zipcode primers and identified by LDR. In Figure 6C, LDR probe sets were designed to identify specific base changes in conversion products. LDR probes anneal in competition with each other to conversion products. Perfectly complementary upstream and downstream LDR probes with no overlap or gap ligate with high specificity. Discriminating probes had different length 5' tails to allow specific product separation on an acrylamide gel. A set of probes used to identify PCR error products in nonconversion of wild-type template is shown.

Figures 7A-B show results of a conversion by natural base and Q₆ convertide. Conversion products from nine templates were detected by PCR/LDR. Each template was a 50-base pair synthetic duplex DNA of identical sequence except for the central four bases which have the sequence indicated. Conversion occurred within these four bases. The expected conversion products

5 produced by starting with the conversion primers having the indicated 3' natural
base or convertide are shown. Figure 7A shows the conversion of the first base to
10 C with and without Q₆ preconversion. Figure 7B shows the conversion of the first
base to T with and without Q₆ preconversion.

5 Figures 8A-B shows conversion by natural base, Q₅, and Q₇
convertides. Conversion products from nine templates were detected by
15 PCR/LDR. Each template was a 50-base pair synthetic duplex DNA of identical
sequence except for the central four bases which have the sequence indicated.
Conversion occurred within these four bases. The expected conversion products
10 produced by starting with the conversion primers having the indicated 3' natural
base or convertide are shown. Figure 8A shows conversion of the first base to G
with and without Q₅ or Q₇ preconversion. Figure 8B shows conversion of the first
base to A with and without Q₅ or Q₇ preconversion.

25 Figures 9A-II show the fidelity of polymerase extension. Primer
15 slippage accounts for many of the observed products of extension (Figure 7 and
Figure 8). In Figure 9A, perfectly complementary primer gives correct product.
For Figure 9B, a T:G mismatch at the second base explains TGGG (or TGCA)
30 product. In Figure 9C, extension from a Q₆:G pairing with no slippage on the
minus strand of the CCGG template (followed by 3' T conversion primers)
20 resulted in the expected TCGA product. Figure 9D shows extension from a Q₆:G
pairing with no slippage on the minus strand of the CTGG template and several
35 other templates (followed by 3' T conversion primers) resulted in the expected
products. In Figure 9E, GG mismatch extension apparently gave the expected GC
product on one template, but perhaps only fortuitously (see Figure 9F). For
40 Figure 9F, all extensions from GG mismatches gave GC extension products,
consistent with a GT mismatch formed by slippage at the preceding base. In
25 Figure 9G, Q₅:G and Q₇:G extension products apparently gave the expected GC
product on one template, but perhaps only fortuitously (see Figure 9H). In Figure
45 9H, all extensions from Q₅:G and Q₇:G mismatches (followed by 3' G conversion
primers) gave GC extension products consistent with a Q₅:T or Q₇:T mismatch at
30 the preceding base.

50 Figure 10A-C is a schematic of the amplification of p53 exon 7
from genomic DNA (Figure 10A), and conversion of the normal MspI site to a

5 *TaqI* site. First, the template is subjected to PCR amplification of the p53 Exon 7
(Figure 10B). Next, a PCR conversion step is carried out, resulting in the
10 conversion of codon 248 to a the to create a (TCGA) *TaqI* restriction site (Figure
10B).

5 Figures 11A-C show the PCR primers used to determine
polymrase fidelity by PCR/RE/LDR at a preexisting restriction site to avoid
15 conversion error. The method used is as described in Figure 6 for conversion
steps. However, in the fidelity experiments shown in Figure 11, the buffer
components were modified, to test for optimal buffer conditions within the
20 conversion process. Figure 11A shows a PCR fidelity assay. A synthetic 50-base
pair duplex marker template (MK) and wild-type p53 exon 7 PCR product are
mixed at known ratios in parallel reactions. Perfect match primers p53-248short
and p53-248shortR amplify the wild-type CCGG and marker CGGG. Then,
25 longer zipcode containing primers p53zip248short and p53zip248shortR were
added. Finally, wild-type was repeatedly digested and reamplified with zipcode
15 primers (Ztop and Zbot). In Figure 11B, preconversion was performed using
primers containing 3' convertide; e.g., p53-248Q₆. Conversion of the *MspI* site to
30 a *TaqI* site with or without preconversion was performed using 3' natural base
primers p53zip248T and p53zip248TR. Long primers were added as above and
20 conversion products further amplified. Wild-type products were digested with the
restriction endonuclease appropriate for the new site. Mutant products were
35 preferentially amplified with zipcode primers. In Figure 11C, LDR probe sets
were designed to query the template sequence around the point of ligation.
Perfectly hybridized upstream and downstream LDR probes with no overlap or
40 gap are preferentially ligated with high specificity. Discriminating probes have
different length 5' tails to allow specific product separation on an acrylamide gel.
Probes shown were used for identification of mutations occurring in the second
45 base of the *MspI* site (no conversion). An extra probe (p53LDR248FTCL) was
used to compare C→T transitions at the first base and second base of the *MspI*
30 site. A comparable set of discriminating and common probes were used to
identify mutations at the second base of the *TaqI* site in conversion products had a
50 T at the 3' penultimate base in the discrimination primers and A at the 5'
penultimate base in the common probe.

Figures 12A-B show the buffer and enzyme dependent PCR errors detected by the PCR/RE/LDR experiments shown in Figure 11. The indicated polymerase/buffer combinations were used to amplify p53 exon 7 from genomic DNA. The same buffers were used in reactions with perfect match primers to reamplify the *MspI* site. In Figure 12A, *Taq* polymerase (T) was used in various test PCR buffers, while in Figure 12B, Vent polymerase (V) was used in various test buffers. Vent polymerase did not amplify p53 exon 7 from genomic DNA in TsK, buffer A, buffer. In this case only, two different enzyme/buffer sets were used for preamplification and "conversion" (not actual conversion, since perfect match primers were used). The AmpliTaq T/TsK exon 7 genomic DNA PCR product was substituted in the Vent V/TsK, buffer A, reamplification. "C" indicates no MK was added (control reaction).

Figure 13 shows the results of a comparison of conversion fidelity. The relative intensities of conversion reaction products is indicated by color and the height of each peak in a 3-D plot. Marker (MK) DNA (with CGGG replacing the *MspI* site) was added at known ratios to wild-type (WT) in parallel reactions. The $-\log(\text{MK:WT})$ indicates relative fraction of MK present, e.g., $-\log(\text{MK:WT})=3$ means the ratio of MK to WT was 1:1000. "C" indicates no MK was added (control reaction). Nonconversion control reactions (C:G) were performed using perfect match 3' C primers. Conversion of the *MspI* site (CCGG) to a *TaqI* site (TCGA) was performed using natural base 3' T primers with and without preconversion using 3' Q₆ nucleotide analog primers (Q₆:G and T:G reactions, respectively). LDR products from *MspI* nonconversion contain CNGG, and products from *TaqI* conversion contain TNGA, but only the central bases (second and third bases) are indicated as _NG_. The LDR products were designed to separate on acrylamide gels by two base differences in size. Some undetermined bands of intermediate size were also observed. Lanes 1-4 were digested with *MspI*, while lanes 5-12 were digested with *TaqI* during PCR/RE/LDR.

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DETAILED DESCRIPTION OF THE INVENTION

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The present invention relates to a method for identifying one or more low abundance sequences differing by one or more single-base changes, insertions, or deletions, from a high abundance sequence, in a plurality of target nucleotide sequences. This method involves a first polymerase chain reaction phase, a second polymerase chain reaction phase, and a restriction endonuclease digestion reaction phase, followed by a third polymerase chain reaction phase and a ligase detection reaction phase.

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10 The starting sample of the present invention is a sample potentially containing one or more low abundance target nucleotide sequences with at least one sequence difference each from the high abundance target sequences present in the sample.

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In the first polymerase chain reaction phase, a primary oligonucleotide primer set is provided. The primary oligonucleotide primer set has a first oligonucleotide primer containing a target-specific portion, and a second oligonucleotide primer containing a target-specific portion. The primary oligonucleotide primers are suitable for hybridization on complementary strands of a corresponding high and low abundance target nucleotide sequences to permit formation of a polymerase chain reaction product. However, the primers each have a mismatch which interferes with formation of such a polymerase chain reaction product when hybridized to any other nucleotide sequence present in the sample. The primary oligonucleotide primers, the sample, and a polymerase are blended to form a primary polymerase chain reaction mixture.

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25 The primary polymerase chain reaction mixture is subjected to two or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment. The denaturation treatment separates hybridized nucleic acid sequences. The hybridization treatment causes the target-specific portions of the primary oligonucleotide primers to hybridize to the target nucleotide sequences. The extension treatment causes the hybridized primary oligonucleotide primers to be extended to form primary extension

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5 products complementary to the target nucleotide sequence to which the primary oligonucleotide primer is hybridized.

10 The efficiency and accuracy of converting the high abundance primary polymerase chain reaction product into a secondary polymerase chain reaction product containing a restriction endonuclease site may be improved by performing a step (referred to a "preconversion") prior to providing the secondary
15 oligonucleotide primer set. This step consists of providing a pre-secondary oligonucleotide primer set having (a) a first oligonucleotide primer, with a target-specific portion, and (b) a second oligonucleotide primer, with a target-specific
20 portion. The target-specific portions are identical or substantially identical to the secondary oligonucleotide primer set but at least one primer contains one or more nucleotide analogs. The oligonucleotides in a particular pre-secondary primer set are suitable for hybridization on complementary strands of the primary extension
25 products. The primary extension product is denatured, and the primary extension products are blended with a polymerase and the pre-secondary oligonucleotide primers to form a pre-secondary polymerase chain reaction mixture. The mixture is subjected to two or more PCR cycles to permit the formation of a pre-secondary
30 polymerase chain reaction product. This product contains one or more nucleotide analogs and opposite strand base changes, which facilitates the conversion of the primary polymerase chain reaction product sequence into a restriction endonuclease recognition site in the subsequent secondary polymerase chain
35 reaction.

40 Next, there is a second polymerase chain reaction phase. This phase involves providing a secondary oligonucleotide primer set having a first oligonucleotide primer with a target-specific portion and a 5' upstream secondary
25 primer-specific portion and a second oligonucleotide primer with a target-specific portion and a 5' upstream secondary primer-specific portion. The secondary oligonucleotide primers in a particular set are suitable for hybridization on
45 complementary strands of the primary extension products to permit formation of a secondary polymerase chain reaction product which contains or creates a restriction endonuclease recognition site when amplifying the high abundance
30 target, but does not contain or create a restriction endonuclease recognition site when amplifying the one or more low abundance targets. The primary extension
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5 products, the secondary oligonucleotide primers, and the polymerase are blended to form a secondary polymerase chain reaction mixture.

10 The secondary polymerase chain reaction mixture is subjected to two or more polymerase chain reaction cycles comprising a denaturation treatment, a hybridization treatment, and an extension treatment. The
5 denaturation treatment involves separation of hybridized nucleic acid sequences. In the hybridization treatment, the secondary oligonucleotide primers hybridize to
15 the primary extension products. The extension treatment causes the hybridized primary extension products to form secondary extension products complementary
20 to the primary extension products. The high abundance secondary extension products contain a restriction site but the low abundance secondary extension products do not.

25 The next phase involves blending a restriction endonuclease with the secondary extension products to form an endonuclease digestion reaction mixture. The restriction endonuclease is one that recognizes and cleaves the
15 restriction endonuclease recognition site within or created when amplifying the high abundance target, but not the low abundance target in the secondary
30 extension products. The restriction endonuclease digestion selectively destroys the high abundance secondary extension products.

20 Next, there is a third polymerase chain reaction phase. This involves providing a tertiary oligonucleotide primer set having a first tertiary
35 primer containing the same sequence as the 5' upstream portion of the first oligonucleotide primer of the secondary oligonucleotide primer set and a second
40 tertiary primer containing the same sequence as the 5' upstream portion of a second oligonucleotide primer of the secondary oligonucleotide primer set. The
25 set of tertiary oligonucleotide primers may be used to amplify all of the secondary extension products. The secondary extension products are blended with the
45 tertiary oligonucleotide primer set, and a polymerase to form a tertiary polymerase chain reaction mixture.

30 The tertiary polymerase chain reaction mixture is subjected to two or more polymerase chain reaction cycles comprising a denaturation treatment, a
50 hybridization treatment, and an extension treatment. The denaturation treatment causes the hybridized nucleic acid sequences to be separated, while the

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hybridization treatment involves hybridization of the tertiary oligonucleotide primers to hybridize to the secondary extension products. During the extension treatment, the hybridized tertiary oligonucleotide primers are extended to form tertiary extension products complementary to the secondary extension products.

5 Next, the tertiary extension products are subjected to a ligase detection reaction. This involves providing a plurality of oligonucleotide probe sets, each set having a first oligonucleotide probe, having a tertiary extension product-specific portion and a detectable reporter label, and a second
15 oligonucleotide probe, having a tertiary extension product-specific portion. The oligonucleotide probes in a particular set are suitable for ligation together when
20 hybridized adjacent to one another on a complementary tertiary extension product-specific portion. However, there is a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence present in the sample.

The tertiary extension products, the plurality of oligonucleotide
25 probe sets, and a ligase are blended to form a ligase detection reaction mixture.

The ligase detection reaction mixture is subjected to one or more
ligase detection reaction cycles having a denaturation treatment, and a
30 hybridization treatment. The denaturation treatment involves separation of hybridized oligonucleotides from the tertiary extension products. In the
20 hybridization treatment, the oligonucleotide probe sets hybridize at adjacent positions in a base-specific manner to their respective tertiary extension products,
35 if present. As a result, adjacent probes ligate to one another to form a ligation product sequence containing the detectable reporter label and the tertiary extension product-specific portions connected together. The oligonucleotide
40 probe sets may hybridize to nucleotide sequences other than their respective complementary tertiary extension products but do not ligate together due to a presence of one or more mismatches and individually separate during the
45 denaturation treatment. Following the ligase detection reaction cycles, the reporter labels of the ligation product sequences are detected which indicates the
30 presence of one or more low abundance target nucleotide sequences in the sample.

Figure 1 is a schematic diagram of the amplification and detection
of mutant DNA by PCR/RE/LDR according to the method of the present
invention. The process begins with a sample containing a normal, or wild-type

sequence in high abundance, and, possibly, a mutant of that sequence, such as a cancer gene, in low abundance. Comparing the mutant and wild type sequences in Figure 1, it is apparent that they differ with respect to only one base. In the wild-type sequence, the distinguishing base is a "C," while the mutant sequence has a "T" at the corresponding location.

First, the sample is subjected to a primary PCR step, which creates a modified target sequence. In the primary PCR procedure shown in Steps 1 and 2 of Figure 1, wild-type and mutant DNA undergo denaturation at 94°C, to create single-stranded DNA templates. As shown in Step 1 of Figure 1, primers A and B, each containing 3' terminal nucleotide analogue are annealed to the single stranded DNA templates. Facilitated by a polymerase, the analogue primers undergo an extension procedure as shown in Step 2 of Figure 1. This process of denaturing double stranded nucleic acids, hybridizing primers to the resulting single stranded nucleic acids, and extending the primers is repeated, in accordance with conventional PCR procedures, to produce, in quantity, extension products in the form of modified target sequences containing the nucleotide analogue. The polymerase chain reaction process is fully described in H. Erlich, et al., "Recent Advances in the Polymerase Chain Reaction," *Science* 252: 1643-50 (1991); M. Innis, et al., *PCR Protocols: A Guide to Methods and Applications*, Academic Press: New York (1990); and R. Saiki, et al., "Primer-directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase," *Science* 239: 487-91 (1988), which are hereby incorporated by reference. Further details for use of PCR in accordance with the present invention are provided in the Examples below.

As shown in Step 1 of Figure 1, the wild-type and mutant sequences have complementary X:W and Y:Z base pairs proximate to the distinguishing bases of these sequences. When these sequences are denatured in the primary PCR procedure, primers A and B are configured to hybridize to those sequences such that Q bonds to the Y and W bases. In subsequent cycles of the primary PCR step, polymerase encounters a Q analog in what is now a template strand. The polymerase can "read" the analog as one of several bases and it will "write" opposite the Q one of several different bases which in effect are "complementary" to the analog. Because different bases can be incorporated at

5 such positions, the products are degenerate, ideally having G, A, T and C present in the same positions opposite Q analogs in the pool of products. These
10 degenerate bases are indicated by "N." As shown in Step 2 of Figure 1, the mismatch, formed in both the wild-type and mutant sequence, is signified as base
5 "N".

15 Nucleotide analogs suitable for the present invention include, but are not limited to, the following: Q1, 1-(2'-deoxy-β-D-ribofuranosyl)imidazole-4-carboxamide; Q2, 1-(2'-deoxy-β-D-ribofuranosyl)-3-nitropyrrole; Q5, 2'-
deoxyinosine; Q6, 6-(2'-deoxy-β-D-ribofuranosyl)-6H,8H-3,4-
20 dihydropyrimido[4,5-c][1,2]oxazine-7-one; Q7, 2-amino-7-(2'-deoxy-β-D-ribofuranosyl)-6-methoxyaminopurine; Q16, 1-(2'-deoxy-β-D-ribofuranosyl)-4-iodopyrazole; Q18, 1-(2'-deoxy-β-D-ribofuranosyl)pyrrole-3-carboxamide; Q19, 1-(2'-deoxy-β-D-ribofuranosyl)-4-nitropyrazole.

25 Next, as shown in Step 3 of Figure 1, a secondary PCR phase is carried out. The oligonucleotide primer set of the secondary PCR phase has a first
15 oligonucleotide primer with a target-specific portion and a 5' upstream secondary primer-specific portion, and a second oligonucleotide primer with a target-specific
30 portion and a 5' upstream secondary primer-specific portion. These are represented by primers "C" and "D" in Step 3 of Figure 1. During the secondary
20 PCR phase, the target specific portions of the secondary PCR primers will hybridize to complementary strands of the primary extension products. As shown
35 in Step 3 of Figure 1, these hybridized primers are then extended using polymerase to form secondary PCR extension products, as shown in Step 3 of
40 Figure 1. The secondary PCR phase is repeated for 2-20 PCR cycles, involving
25 denaturation of double stranded nucleic acids, primer hybridization, and primer extension, until the primary PCR extension product is sufficiently amplified.

45 Due to the "T" v. "C" difference in the mutant sequence versus the wild-type sequence, respectively, as shown in Figure 1, the secondary extension
products can be treated with the appropriate restriction endonuclease (RE) that
30 recognizes the RE site in the secondary extension products derived from the wild-type sequence. However, the secondary PCR extension product formed from the
50 mutant nucleic acid sequence does not contain a restriction endonuclease site. In the embodiment of Figure 1, the restriction endonuclease recognition site

5 incorporated in the secondary extension products derived from the wild-type
sequence is a *TaqI* recognition site, 5' -TCGA-3'. *TaqI* cleaves specifically
10 within this recognition site, between the T and the C, in each strand of the double
stranded DNA secondary extension product. However, other restriction
5 endonuclease sites and their corresponding restriction endonucleases could instead
be utilized.

15 Once the restriction site is incorporated into the high abundance
target, the appropriate restriction endonuclease is added, under conditions that
allow the digestion of the restriction site nucleotide sequence. Restriction
20 endonucleases, derived from bacteria, are enzymes that cut DNA within a
nucleotide chain. Each restriction endonuclease recognizes specific short
oligonucleotides from four to eight residues long in a DNA sequence. Under
appropriate conditions the RE cleaves the each strand at a phosphodiester bond
25 within the recognition site. A restriction endonuclease cuts a pure DNA sample
into consistently reproducible fragments that can easily be separated by gel
electrophoresis. Several hundred restriction endonucleases have been identified
and are commercially available. See Darnell et al., "Manipulating
30 Macromolecules," Molecular Cell Biology, Second Edition, New York, New
York: W. H. Freeman and Company, pp. 189-225 (1990), which is hereby
20 incorporated by reference. Any RE can be used in accordance with the method of
the present invention. The choice of RE to be used is made based upon the
information available for the DNA in the starting sample. The RE sites that occur
within a gene are readily available through resources such as GenBank; the
40 manufacturer, if the DNA has been obtained commercially, or will have been
identified as part of the study of the gene, prior to the use of the method of the
present invention. Primer design for the PCR phases of the invention is based
upon the knowledge of the restriction sites in a gene. RE site identification can be
45 readily determined by DNA mapping and DNA cloning. Watson et al., "In Vitro
Mutagenesis," Recombinant DNA, Second Edition, New York, New York: W. H.
30 Freeman and Company, pp. 191-194 (1983), which is hereby incorporated by
reference. Optimal conditions for complete cleavage by a restriction
endonuclease are specific. Digestion should be carried out per manufacturer's
50 recommendations for a particular RE, or as described in Sambrook et al.,

Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Press, NY (1989), which is hereby incorporated by reference.

After treatment of the secondary extension product with a restriction endonuclease, a tertiary PCR process is carried out. As shown in Step 6 of Figure 1, this involves providing a tertiary oligonucleotide primer set having a first tertiary primer that is a universal primer (U1), containing the same sequence as the 5' upstream portion of the first oligonucleotide primer of the secondary oligonucleotide primer set, and a second tertiary primer containing the same sequence as the 5' upstream portion of a second oligonucleotide primer of the secondary oligonucleotide primer set. The set of tertiary oligonucleotide primers are used in amplifying the restriction endonuclease-digested secondary extension products. The secondary extension products are blended with the tertiary oligonucleotide primer set, and a polymerase to form a tertiary polymerase chain reaction mixture.

The tertiary polymerase chain reaction mixture is subjected to polymerase chain reaction conditions involving denaturation of double stranded nucleic acids, hybridization of tertiary oligonucleotide primers to the resulting single stranded nucleic acids, and extension of the hybridized primer. This procedure is repeated through a sufficient number of cycles involving these steps in order to produce an appropriate amount of tertiary PCR extension products. Since the secondary PCR extension products have been treated with a restriction endonuclease, the tertiary PCR extension products derived from the wild-type nucleic acid sequence are short as a result of their being cleaved proximate to the nucleotide corresponding to the distinguishing base. On the other hand, the tertiary PCR extension products derived from the mutant nucleic acid sequence are larger and not cleaved proximate to the nucleotide corresponding to the distinguishing base. See Figure 1, Step 6. As a result, the tertiary extension products derived from the mutant nucleic acid sequence are readily distinguished from those derived from wild-type nucleic acid sequence in the subsequent LDR step.

A second restriction endonuclease digest may also be carried out following the tertiary PCR phase, to remove any high abundance targets that may have been amplified due to PCR error or incomplete digestion in the first RE step.

Following the tertiary PCR extension procedure, the resulting tertiary extension products are subjected to an LDR procedure according to either Step 7A or Step 7B of Figure 1. In either of these alternatives, the LDR procedure begins by first denaturing the tertiary PCR extension products to produce single-stranded DNA (ssDNA). The discrimination probes are distinguished by having a different nucleotide on the 3' ends and a different number of adenine bases on their 5' ends. As a result, ligation products produced from each of the discrimination probes are identified by detection of the fluorescent label and distinguished from one another on a gel by their differing electrophoretic mobility.

In Step 7A of Figure 1, the LDR probe set contains 4 alternative discrimination probes and a common probe, which can ligate to any of the four discrimination probes. The common probe contains a fluorescent tag on its 3' end. The tertiary extension products, these oligonucleotide probes, and a thermostable ligase are combined to form a ligase detection reaction mixture and subjected to a series of ligase detection reaction cycles.

The ligase detection reaction is described generally in WO 90/17239 to Barany et al., "Cloning, Overexpression and Nucleotide Sequence of a Thermostable DNA Ligase-encoding Gene," *Gene*, 109:1-11 (1991), and F. Barany, "Genetic Disease Detection and DNA Amplification Using Cloned Thermostable Ligase," *Proc. Natl. Acad. Sci. USA*, 88:189-193 (1991), and U.S. Patent Nos. 5,494,810, 5,830,711 and 6,027,889 to Barany, the disclosures of which are hereby incorporated by reference. In accordance with the present invention, the ligase detection reaction can use 2 sets of complementary oligonucleotides. This is known as the ligase chain reaction, which is described in the immediately preceding references, which are hereby incorporated by reference. Alternatively, the ligase detection reaction can involve a single cycle that is known as the oligonucleotide ligation assay. See Landegren, et al., "A Ligase-Mediated Gene Detection Technique," *Science* 241:1077-80 (1988); Landegren, et al., "DNA Diagnostics -- Molecular Techniques and Automation," *Science* 242:229-37 (1988); and U.S. Patent No. 4,988,617 to Landegren, et al., which are hereby incorporated by reference.

During ligase detection reaction phases, the denaturation treatment is carried out at a temperature of 80-105° C, while hybridization takes place at 50-

5 85° C. Each cycle comprises a denaturation treatment and a thermal hybridization
treatment which in total is from about one to five minutes long. Typically, the
10 ligation detection reaction involves repeatedly denaturing and hybridizing for 2 to
50 cycles. The total time for the ligase detection reaction phase is 1 to 250
5 minutes.

The preferred thermostable ligase is that derived from *Thermus*
15 *aquaticus*. This enzyme can be isolated from that organism. M. Takahashi, et al.,
"Thermophilic DNA Ligase," *J. Biol. Chem.* 259:10041-47 (1984), which is
hereby incorporated by reference. Alternatively, it can be prepared
20 recombinantly. Procedures for such isolation as well as the recombinant
production of *Thermus aquaticus* ligase (as well as *Thermus thermophilus* ligase)
are disclosed in WO 90/17239 to Barany, et al., F. Barany, et al., "Cloning,
Overexpression and Nucleotide Sequence of a Thermostable DNA-Ligase
25 Encoding Gene," *Gene* 109:1-11 (1991), and U.S. Patent Nos. 5,494,810 and
15 5,830,711 to Barany, which are hereby incorporated by reference. These
references contain complete sequence information for this ligase as well as the
encoding DNA. Other suitable ligases include *E. coli* ligase, T4 ligase, and
30 *Pyrococcus* ligase.

The ligation detection reaction mixture may include a carrier DNA,
20 such as salmon sperm DNA.

The hybridization step in the ligase detection reaction, which is
35 preferably a thermal hybridization treatment, discriminates between nucleotide
sequences based on a distinguishing nucleotide at the ligation junctions. The
difference between the target nucleotide sequences can be, for example, a single
40 nucleic acid base difference, a nucleic acid deletion, a nucleic acid insertion, or
25 rearrangement. Such sequence differences involving more than one base can also
be detected.

The oligonucleotide probe sets, as noted above, may have a
45 reporter label suitable for detection. Useful labels include chromophores,
30 fluorescent moieties, enzymes, antigens, heavy metals, magnetic probes, dyes,
phosphorescent groups, radioactive materials, chemiluminescent moieties, and
50 electrochemical detecting moieties. Also appropriate would be the use of
molecular weight tags for discrimination by mass spectroscopy, such as a matrix

5 assisted laser desorption ionization – time of flight mass spectroscopy (MALDI-TOF) array system.

10 The oligonucleotide probe sets can be in the form of ribonucleotides, deoxynucleotides, modified ribonucleotides, modified deoxyribonucleotides, peptide nucleotide analogues, modified peptide nucleotide analogues, modified phosphate-sugar-backbone oligonucleotides, nucleotide
15 analogs, and mixtures thereof.

The oligonucleotides of the oligonucleotide probe sets each have a hybridization or melting temperature (i.e. T_m) of 66-70°C. These oligonucleotides
20 are 20-28 nucleotides long.

As shown in Step 7A of Figure 1, the ligation products derived from the mutant nucleic acid sequence are formed from the discrimination probe having 2 adenine bases on its 5' end. The other discrimination probes will not be
25 ligated to the common probe as a result of the mutant nucleic acid sequence being present.

On the other hand, for the most part, no ligation products using the above-described probe sets, will be formed from the wild-type sequence. This occurs because the endonuclease digestion causes tertiary extension products derived from the wild-type nucleic acid sequence to be cleaved, and, as a result,
30 both the common and discrimination probes of that probe set will not hybridize to such tertiary extension product in a manner permitting them to be ligated together. However, to the extent the endonuclease digestion of the secondary extension products derived from the wild-type sequence is incomplete, a small residual amount of uncleaved tertiary extension product derived from the wild-type
35 sequence may be present in the ligase detection reaction mixture. This will cause ligation products to be produced from the discrimination probe having no adenines at its 5' end.

45 Figure 2 shows the gel electrophoresis detection of products formed from the ligase detection reaction procedure of Figure 1, Step 7A. As shown in Figure 2, unligated probes have great electrophoretic mobility and form a band at the bottom of the gel. Ligation products derived from any small amount of tertiary extension products derived from the wild-type sequence have the next
50 greatest electrophoretic mobility and form a band at an intermediate location on

the gel. Ligation products derived from the mutant sequence have less electrophoretic mobility than the wild-type ligation products, and form a thick band slightly above that of the wild-type ligation products. If no mutant sequence is present in the sample, no such gel band will be formed. The top two bands are trace ligation products with low electrophoretic mobility. These high molecular weight bands are artifacts, presumably arising from polymerase error and template decomposition during PCR. The composition of the PCR buffer can strongly affect the fraction of these PCR error products present in the sample after amplification.

The use of capillary and gel electrophoresis to detect DNA products based on differences in their electrophoretic mobility is well known. See e.g., Grossman, et al., "High-density Multiplex Detection of Nucleic Acid Sequences: Oligonucleotide Ligation Assay and Sequence-coded Separation," Nucl. Acids Res. 22(21): 4527-34 (1994), which is hereby incorporated by reference.

As an alternative to the ligase detection reaction procedure shown in Figure 1, Step 7B, the discrimination probes can be provided with different address-specific portions (i.e. ZIP1, ZIP2, ZIP3, and ZIP4) at their 5' ends. This enables the ligation products to be detected on an addressable array having different capture oligonucleotide probes which are complementary to the address-specific portions on the discrimination probes. As a result, each ligation product is directed to a different address of a DNA microarray during a hybridization procedure subsequent to the ligase detection reaction phase. The hybridized ligation products all have the same label but are distinguished from one another by the location on which the ligation products are immobilized. Alternatively, different labels can be on the discrimination base, while the address-specific portion is on the common probe. In this embodiment, different ligation products will be immobilized on the array at locations with the same capture probe; however, the different ligation products will be distinguished by different labels.

Figure 3 shows the detection of ligation products resulting from the process of Figure 1, Step 7B using an addressable array. In this case, as shown in Figure 1, Step 7B, the common probe has a label and the discrimination probes have the address-specific portion. In Figure 3, the ligation products are caused to

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contact the addressable array under conditions effective to permit address-specific portions of the ligation products to hybridize to capture oligonucleotides on the array. As shown in Figure 3, Steps 2 and 3, discrimination probes (e.g., probes having address-specific portions ZIP4 (not shown) and ZIP3 (shown)) which do not form ligation products are immobilized on the array but are not detected due to the absence of a label. Unligated common probes do not hybridize to the array and are subsequently washed away (e.g., at 65°C-80°C and low salt) so they produce no signal. Any small amount of ligation products derived from the wild-type nucleic acid sequence (due to incomplete upstream endonuclease digestion) are immobilized (together with any of the corresponding unligated discrimination probe) at the capture oligonucleotide complementary to address-specific portion ZIP1. Similarly, any ligation products derived from the mutant sequence are immobilized (together with any of the corresponding unligated probe) at the capture oligonucleotide complementary to address-specific portion ZIP2. The presence of mutant sequence and/or wild-type sequence is detected in this embodiment by the existence of a fluorescent signal at respective different locations on the array. Heterozygosity is indicated by equal signals at the capture oligonucleotides complementary to address-specific portions ZIP1 and ZIP2. The signals may be quantified using a fluorescent imager. This format uses a unique address for each allele and may be preferred for achieving very accurate detection of low levels of signal (30 to 100 attomoles of LDR product).

The use of a solid support with an array of capture oligonucleotides is fully disclosed in pending provisional U.S. Patent Application Serial No. 60/011,359, which is hereby incorporated by reference. This method involves providing a solid support having an array of positions each suitable for attachment of an oligonucleotide. A linker or support (preferably non-hydrolyzable), suitable for coupling an oligonucleotide to the solid support at each of the array positions, is attached to the solid support. An array of oligonucleotides on a solid support is formed by a series of cycles of activating selected array positions for attachment of either single or a multiple nucleotides at the activated array positions.

When using such arrays, the oligonucleotide probes used in the above-described coupled PCR and LDR phases, respectively, have an addressable array-specific portion. After the PCR and LDR phases are completed, the

5 addressable array-specific portions for the products of such processes remain
single stranded and are caused to hybridize to the capture oligonucleotides during
10 a capture phase. C. Newton, et al., "The Production of PCR Products With 5'
Single-Stranded Tails Using Primers That Incorporate Novel Phosphoramidite
5 Intermediates," Nucl. Acids Res. 21(5):1155-62 (1993), which is hereby
incorporated by reference.

15 During the capture phase of the process, the mixture is contacted
with the solid support at a temperature of 45-90° C and for a time period of up to
60 minutes. Adding cations, volume exclusion or chaotropic agents may
20 accelerate hybridizations. When an array consists of dozens to hundreds of
addresses, it is important that the correct ligation product sequences have an
opportunity to hybridize to the appropriate address. This may be achieved by the
25 thermal motion of oligonucleotides at the high temperatures used, by mechanical
movement of the fluid in contact with the array surface, or by moving the
15 oligonucleotides across the array by electric fields. After hybridization, the array
is washed sequentially with a low stringency wash buffer and then a high
stringency wash buffer.

30 It is important to select capture oligonucleotides and addressable
nucleotide sequences which will hybridize in a stable fashion. This requires that
20 the oligonucleotide sets and the capture oligonucleotides be configured so that the
oligonucleotide sets hybridize to the target nucleotide sequences at a temperature
35 less than that which the capture oligonucleotides hybridize to the addressable
array-specific portions. Unless the oligonucleotides are designed in this fashion,
false positive signals may result due to capture of adjacent unreacted
40 25 oligonucleotides from the same oligonucleotide set which are hybridized to the
target.

45 The capture oligonucleotides can be in the form of ribonucleotides,
deoxyribonucleotides, modified ribonucleotides, modified deoxyribonucleotides,
peptide nucleotide analogues, modified peptide nucleotide analogues, modified
30 phosphate-sugar backbone oligonucleotides, nucleotide analogues, and mixtures
thereof.

50 It may be desirable to destroy chemically or enzymatically
unconverted all unligated LDR oligonucleotide probes that contain addressable

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5 nucleotide array-specific portions prior to capture of the ligation products on a
DNA array. Such unconverted probes will otherwise compete with ligation
10 products for binding at the addresses on the array of the solid support which
contain complementary sequences. Destruction can be accomplished by utilizing
5 an exonuclease, such as exonuclease III (L-H Guo and R. Wu, Methods in
Enzymology 100:60-96 (1985), which is hereby incorporated by reference) in
15 combination with LDR probes that are blocked at the ends and not involved with
ligation of probes to one another. The blocking moiety could be a reporter group
or a phosphorothioate group. T.T. Nikiforow, et al., "The Use of
20 Phosphorothioate Primers and Exonuclease Hydrolysis for the Preparation of
Single-stranded PCR Products and their Detection by Solid-phase Hybridization,"
PCR Methods and Applications, 3:p.285-291 (1994), which is hereby incorporated
by reference. After the LDR process, unligated probes are selectively destroyed
25 by incubation of the reaction mixture with the exonuclease. The ligated probes
are protected due to the elimination of free 3' ends which are required for initiation
15 of the exonuclease reaction. This approach results in an increase in the signal-to-
noise ratio, especially where the LDR reaction forms only a small amount of
product. Since unligated oligonucleotides compete for capture by the capture
30 oligonucleotide, such competition with the ligated oligonucleotides lowers the
signal. An additional advantage of this approach is that unhybridized label-
20 containing sequences are degraded and, therefore, are less able to cause a target-
independent background signal, because they can be removed more easily from
35 the DNA array by washing.

40 A wide variety of infectious diseases can be detected by the
25 method of the present invention. Typically, these are caused by bacterial, viral,
parasite, and fungal infectious agents. The resistance of various infectious agents
to drugs can also be determined using the present invention.

45 Bacterial infectious agents which can be detected by the present
invention include *Escherichia coli*, *Salmonella*, *Shigella*, *Klebsiella*,
30 *Pseudomonas*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*,
Mycobacterium avium-intracellulare, *Yersinia*, *Francisella*, *Pasteurella*, *Brucella*,
50 *Clostridia*, *Bordetella pertussis*, *Bacteroides*, *Staphylococcus aureus*,
Streptococcus pneumoniae, *B-Hemolytic strep.*, *Corynebacteria*, *Legionella*.

5 *Mycoplasma*, *Ureaplasma*, *Chlamydia*, *Neisseria gonorrhea*, *Neisseria*
meningitides, *Hemophilus influenza*, *Enterococcus faecalis*, *Proteus vulgaris*,
10 *Proteus mirabilis*, *Helicobacter pylori*, *Treponema palladium*, *Borrelia*
burgdorferi, *Borrelia recurrentis*, *Rickettsial pathogens*, *Nocardia*, and
5 *Actinomycetes*.

Fungal infectious agents, which can be detected by the present
15 invention, include *Cryptococcus neoformans*, *Blastomyces dermatitidis*,
Histoplasma capsulatum, *Coccidioides immitis*, *Paracoccidioides brasiliensis*,
Candida albicans, *Aspergillus fumigatus*, *Phycomycetes (Rhizopus)*, *Sporothrix*
20 *schenckii*, *Chromomycosis*, and *Maduromycosis*.

Viral infectious agents, which can be detected by the present
invention, include human immunodeficiency virus, human T-cell
lymphocytotropic virus, hepatitis viruses (e.g., Hepatitis B virus and Hepatitis C
25 virus), Epstein-Barr virus, cytomegalovirus, human papillomaviruses, orthomyxo
15 viruses, paramyxo viruses, adenoviruses, corona viruses, rhabdo viruses,
polioviruses, toga viruses, bunya viruses, arena viruses, rubella viruses, and reo
viruses.

Parasitic agents which can be detected by the present invention
include *Plasmodium falciparum*, *Plasmodium malaria*, *Plasmodium vivax*,
20 *Plasmodium ovale*, *Onchocerca volvulus*, *Leishmania*, *Trypanosoma spp.*,
35 *Schistosoma spp.*, *Entamoeba histolytica*, *Cryptosporidium*, *Giardia spp.*,
Trichomonas spp., *Balantidium coli*, *Wuchereria bancrofti*, *Toxoplasma spp.*,
Enterobius vermicularis, *Ascaris lumbricoides*, *Trichuris trichiura*, *Dracunculus*
medinensis, trematodes, *Diphyllbothrium latum*, *Taenia spp.*, *Pneumocystis*
40 *carinii*, and *Necator americanus*.

The present invention is also useful for detection of drug resistance
by infectious agents. For example, vancomycin-resistant *Enterococcus faecium*,
45 methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus*
pneumoniae, multi-drug resistant *Mycobacterium tuberculosis*, and AZT-resistant
30 human immunodeficiency virus can all be identified with the present invention.

Genetic diseases can also be detected by the method of the present
invention. This can be carried out by prenatal or post-natal screening for
50 chromosomal and genetic aberrations or for genetic diseases. Examples of

5 detectable genetic diseases include: 21 hydroxylase deficiency, cystic fibrosis,
Fragile X Syndrome, Turner Syndrome, Duchenne Muscular Dystrophy, Down
10 Syndrome or other trisomies, heart disease, single gene diseases, HLA typing,
phenylketonuria, sickle cell anemia, Tay-Sachs Disease, thalassemia, Klinefelter
5 Syndrome, Huntington Disease, autoimmune diseases, lipidosis, obesity defects,
hemophilia, inborn errors of metabolism, and diabetes.

15 Cancers that can be detected by the method of the present invention
generally involve oncogenes, tumor suppressor genes, or genes involved in DNA
amplification, replication, recombination, or repair. Examples of these include:
10 BRCA1 gene, p53 gene, APC gene, Her2/Neu amplification, Bcr/Ab1, K-ras
gene, and human papillomavirus Types 16 and 18. Various aspects of the present
invention can be used to identify amplifications, large deletions as well as point
25 mutations and small deletions/insertions of the above genes in the following
common human cancers: leukemia, colon cancer, breast cancer, lung cancer,
15 prostate cancer, brain tumors, central nervous system tumors, bladder tumors,
melanomas, liver cancer, osteosarcoma and other bone cancers, testicular and
ovarian carcinomas, head and neck tumors, and cervical neoplasms.

30 In the area of environmental monitoring, the present invention can
be used for detection, identification, and monitoring of pathogenic and indigenous
20 microorganisms in natural and engineered ecosystems and microcosms such as in
municipal waste water purification systems and water reservoirs or in polluted
35 areas undergoing bioremediation. It is also possible to detect plasmids containing
genes that can metabolize xenobiotics, to monitor specific target microorganisms
in population dynamic studies, or either to detect, identify, or monitor genetically
40 modified microorganisms in the environment and in industrial plants.

45 In the food and feed industry, the present invention has a wide
variety of applications. For example, it can be used for identification and
characterization of production organisms such as yeast for production of beer,
wine, cheese, yogurt, bread, etc. Another area of use is with regard to quality
30 control and certification of products and processes (e.g., livestock, pasteurization,
and meat processing) for contaminants. Other uses include the characterization of
50 plants, bulbs, and seeds for breeding purposes, identification of the presence of
plant-specific pathogens, and detection and identification of veterinary infections.

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The present invention is useful in detecting the presence of one or more of a low abundance sequence, that differs from a high abundance sequence by one or more single-base changes, insertions, deletions, or translocations when the low abundance sequence is present in the same sample in less than a 1:1,000 molar ratio, preferably, in less than a 1:10,000 molar ratio, and, most preferably, in less than a 1:100,000 molar ratio, to the amount of the high abundance sequence.

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Another aspect of the present invention is its capability to quantify the amount of target nucleotide sequence in a sample. This can be achieved by establishing an internal standard (i.e. where the standard establishing material is amplified and detected with the sample).

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For quantification using an internal standard, a known amount of one or more marker target nucleotide sequences is added to the sample. In addition, a plurality of marker-specific oligonucleotide probe sets are added along with the ligase, the previously discussed oligonucleotide probe sets, and the tertiary extension products to a mixture. The marker-specific oligonucleotide probe sets have (1) a first oligonucleotide probe with a target-specific portion complementary to the marker target nucleotide sequence, and (2) a second oligonucleotide probe with a target-specific portion complementary to the marker target nucleotide sequence and a detectable reporter label. The oligonucleotide probes in a particular marker-specific oligonucleotide set are suitable for ligation together when hybridized adjacent to one another on a corresponding marker target nucleotide sequence. However, there is a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence present in the sample or added marker sequences. The presence of ligation product sequences is identified by detection of reporter labels. The amount of target nucleotide sequences in the sample is then determined by comparing the amount of ligation product sequence generated from known amounts of marker target nucleotide sequences with the amount of other ligation product sequences.

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The present invention also relates to a kit for identifying one or more low abundance sequences differing by one or more single-base changes, insertions, or deletions, from a high abundance sequence in a plurality of target nucleotide sequences. This kit provides a primary oligonucleotide primer set, a

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secondary oligonucleotide primer set, a tertiary oligonucleotide primer set, and a plurality of oligonucleotide probe sets.

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The primary oligonucleotide primer set provided in the kit has (a) a first oligonucleotide primer containing a target-specific portion, and (b) a second oligonucleotide primer containing a target-specific portion. The primary oligonucleotide primers are suitable for hybridization on complementary strands of a corresponding high and low abundance target nucleotide sequences to permit formation of a primary extension product. However, the primers have a mismatch which interferes with formation of such a polymerase chain reaction product when hybridized to any other nucleotide sequence present in the sample.

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The secondary oligonucleotide primer set provided in the kit has (a) a first oligonucleotide primer, containing a target-specific portion and a 5' upstream secondary primer-specific portion, and (b) a second oligonucleotide primer, containing a target-specific portion and a 5' upstream secondary primer-specific portion. The secondary oligonucleotide primers are suitable for hybridization on complementary strands of the primary extension products to permit formation of a secondary extension product which contains or creates a restriction endonuclease recognition site when amplifying the high abundance target, but does not contain or create a restriction endonuclease recognition site when amplifying the one or more low abundance targets.

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The tertiary oligonucleotide primer set provided in the kit have (a) a first tertiary primer containing the same sequence as the 5' upstream portion of the first oligonucleotide primer of the secondary oligonucleotide primer set, and (b) a second tertiary primer containing the same sequence as the 5' upstream portion of a second oligonucleotide primer of the secondary oligonucleotide primer set. The set of tertiary oligonucleotide primers may be used to amplify all of the secondary extension products.

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The kit also provides a plurality of oligonucleotide probe sets. Each set has (a) a first oligonucleotide probe, containing a tertiary extension product-specific portion and a detectable reporter label, and (b) a second oligonucleotide probe, containing a tertiary extension product-specific portion. The oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a complementary tertiary extension

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product-specific portion. However, the probes have a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence present in the sample.

The kit may also contain a polymerase, an endonuclease restriction enzyme, and/or a ligase, as described above.

EXAMPLES

Optimizing Analog Additions under PCR Conditions

Example 1 - Oligonucleotide Synthesis for Analog Efficiency

Oligonucleotides were synthesized at the 0.2 μ mole scale by cyanoethyl phosphoramidite chemistry on an Applied Biosystems 394 DNA synthesizer. Standard 500Å CPG columns and reagents (Applied Biosystems) were used with the following exceptions: oligonucleotides 50 bases in length were synthesized using wide-pore 1000Å CPG columns (Applied Biosystems); oligonucleotides with fluorescent dye FAM at the 5' terminus were synthesized using FAM phosphoramidite (Applied Biosystems) with a 15 minute coupling step; oligonucleotides with 5' phosphate were synthesized using phosphorylation reagent (Glen Research), with a 15 minute coupling step; oligonucleotides with 3' blocking group were synthesized using Y-Spacer CPG columns (Glen Research). Oligonucleotides with the 3' nucleotide analogs 2'-deoxyinosine Q₅, 6-(2'-deoxy- β -D-ribofuranosyl)-6H, 8H-3,4-dihydropyrimido[4,5-c][1,2]oxazine-7-one (Q₆), and 2-amino-7-(2'-deoxy- β -D-ribofuranosyl)-6-methoxyaminopurine Q₇ were synthesized using 2'-deoxyinosine-CPG, dP-CPG and dK-CPG, respectively (Glen Research), as shown in Figure 5. The oligonucleotide primers containing Q₁, Q₂ and Q₁₈ at the Y-position were synthesized from Q₁, Q₂, and Q₁₈ derived-CPG synthesized from Q₁ (Johnson et al., "The Synthesis and Stability of Oligodeoxyribonucleotides Containing the Deoxyadenosine Mimic 1-(2'-Deoxy-Beta-D-Ribofuranosyl)imidazole-4-Carboxamide," *Nucleic Acid Res.*, 25:559-67 (1997)), Q₂ (Bergstrom et al., *Journal of the American Chemical Society*,

117:1201-9 (1995)) and Q₁₈ (Zhang et al., "Exploratory Studies on Azole Carboxamides as Nucleobase Analogs: Thermal Denaturation Studies on Oligodeoxyribonucleotide Duplexes Containing Pyrrole-3-Carboxamide," Nucleic Acids Res., 26:2208-15 (1998)) by the method of Pon et al. (Pon et al., "Derivatization of Controlled Pore Glass Beads for Solid Phase Oligonucleotide Synthesis," BioTechniques, 6:768-75 (1988)).

Example 2 - PCR Polymerases and Buffers

The DNA polymerases used were *AmpliTaq*, *AmpliTaq* - Stoeffel Fragment, *AmpliTaq* - Fluorescent Sequencing (Applied Biosystems), *Vent* and *Vent(exo')* (New England Biolabs), and *Expand* polymerase mix (*Taq* and *Pfu* polymerase mixture, in *Expand High Fidelity* kit, Boehringer Mannheim). The commercially available PCR buffers used were supplied in the *AmpliTaq* and *Expand High Fidelity* kits. An alternative buffer C:NF, buffer G(f), is described elsewhere (Day et al., Nucleic Acids Res., (1999)). Briefly, C:NF reactions contain 20 mM citrate pH 7.6, 200 µg/ml bovine serum albumin, 2.5 mM MgCl₂, 200 µM dNTP (each) and either 16 mM (NH₄)₂SO₄, or 50 mM potassium acetate, 10% formamide, primers, and template DNA. All PCR and LDR reactions described below were performed under paraffin oil.

Example 3 - Mismatch Extension Efficiency

Primers containing natural bases and nucleotide analogs were used in PCR to compare and measure the efficiency of product formation from synthetic duplex p53 exon 7 templates having *MspI* (CCGG), *TaqI* (TCGA), *HhaI* (GCGC), c. *Tail* (ACGT) sites at the *MspI* position containing codon 248. The primers hybridized to wild-type sequence on either side of the *MspI* site with the 3' ends of the primers extending one base into the site on each side, see Figure 6A. Eight different analogs and the four natural bases were tested in parallel reactions on each of the four synthetic templates. PCR was performed using Stoeffel *Taq* or *Taq*-Fluorescent Sequencing polymerases with the buffer supplied by manufacturer for each polymerase. 10 pmol of each primer and 20

5 fmol of duplex template were used, and 0.2 mM each dNTP and 4 mM MgCl₂.
Parallel reactions underwent 10, 20, 30, 40, and 50 PCR cycles of 94°C for 15 sec,
10 65°C for 1 min. Efficiency and yield were determined from samples run on 6%
agarose gels and stained with ethidium bromide.

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15 **Example 4 - Mismatch Conversion Product Sequencing**

Products most efficiently amplified by each analog were diluted
1000-fold in water. The diluted DNA products were reamplified for 20 cycles of
20 94°C for 15 sec, 65°C for 2 min. using the same polymerase and buffer as in the
previous PCR, but with the addition of 10 pmol of "zipcode" containing primers
p53zip248 and p53zip248, shown in Figure 6A. Zipcode sequences are
oligonucleotides with no known sequence similarity to DNA sequences in any
25 organism. Amplification with zipcode primers is intended to specifically amplify
the zipcode containing products of the previous PCR; *i.e.*, only converted DNA
(containing zipcodes) and not the nearly identical unconverted DNA (lacking
zipcodes) will be amplified. Conversion products were run on 8% agarose gels
30 and bands of the expected size excised. DNA was extracted from the gel slices by
centrifugation in a 235C microcentrifuge (Fisher) for 30 min through 0.45 µm
20 HVLP filter (Millipore). The conversion product was dried and resuspended in
ABI Dye Terminator Cycle Sequencing reaction mix with one of the zipcode
35 primers according to kit instructions (Applied Biosystems). An equal volume (3
µl each) of sequencing reaction was combined with dye mix consisting of 83%
formamide (Eastman), 4 mM EDTA and 8 mg/ml Blue Dextran (Sigma). Samples
40 were electrophoresed on a 7M urea, 10% acrylamide gel (19:1 bis, 0.6x TBE in
gel and running buffer) in an ABI 373 DNA Sequencer. Data were analyzed
25 using ABI 373A DNA Sequencer Data Analysis software version 1.2.0.

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30 **Example 5 - Conversion Product Identification, With and Without A
Preconversion Step**

50 Conversion fidelity was tested using nine different synthetic
templates, with and without preconversion using three primers containing Q₅, Q₆,

and Q₇ (see Example 1). Preconversion PCR was performed with 3' analog primers prior to adding the desired natural base primers, in an effort to avoid mismatch primer extension. The 50-basepair duplex DNA templates contained the wild-type p53 sequence surrounding codon 248, shown in Figure 6B, except for the bases corresponding to the *MspI* site (CCGG). The following sequences were substituted at the *MspI* position: 1) CCGG (wild-type), 2) CTGG, 3) CGGG, 4) CAGG, 5) TCGA, 6) GCGC, 7) ACGT, 8) ACGT, and 9) GCGC. A pre-secondary PCR reaction ("preconversion") was performed with hot start using 50 fmol/ μ l p53-248Q_N and p53-248Q_NR primers, and Vent (exo-) in CiNF buffer, buffer G(I), and 10 fmol/ μ l of duplex template. Preconversion used 2 PCR cycles of 94°C 15 sec, 55°C 1 min, 60°C 1 min. Product was stored at 4°C. Conversion reactions were started with 1 μ l of preconversion reaction containing the same polymerase and buffer, but no additional template. Each reaction required 10 pmol of each primer, using one of the four pairs p53zip248 \underline{N} and p53zip248 \underline{NR} (\underline{N} = C, T, G, or A). Parallel conversion reactions with no preconversion were initiated with a hot start by adding 10 fmol of synthetic duplex template instead of preconversion reaction aliquot. PCR cycles were as follows: 5 cycles of 94°C 15 sec, 55°C + 1° per cycle 1 min, 60°C 1 min; then 20 cycles of 94°C 15 sec, and 60°C 2 min. A final extension was performed at 60°C for 5 min. Polymerase was inactivated by freezing and thawing twice. Products were diluted 10x in water and reamplified by adding 1 μ l to 20 μ l of Expand polymerase and buffer mix. PCR was performed for 20 cycles (30 cycles for low yield reactions) of 94°C 15 sec, 65°C 2 min using 12 pmol of zipcode primers Ztop and Zbot (Figure 6). LDR was performed as described below to identify the conversion products generated.

Example 6 - Ligase Detection Reaction

Ligase detection reactions were performed in standard LDR buffer (25 mM Tris pH 7.6, 12 mM MgCl₂, 65 μ g/ml bovine serum albumin, 100 mM KCl, and 10 mM DTT). Each 20 μ l reaction contained approximately 500 fmol of dsDNA (1 μ l of sample), 500 fmol of each discriminating probe, and 750 fmol of common probe, as shown in Figure 6C. Sets of discriminating and common

5 probes were synthesized to perform LDR on the expected conversion products and
varied at the bases (B_i) corresponding to the *MspI* position sense strand ($B_1B_2B_3B_4$
10 = CCGG for wild-type). The discrimination probes had wild-type sequence and
terminate in $-B_1B_2(-OH\ 3')$. The discrimination probes were synthesized as a set
5 of four probes each with C, T, G and A in turn at B_2 . The common LDR probes
had $(5'PO_4-)B_3B_4-$ followed by wild-type sequence, and hybridized to the
15 template with its 5' base adjacent to the 3' base of a discrimination probe.
Discrimination probes varied the 3' terminal base to identify error products at B_2
of the *MspI* position. For simplicity, only B_2 was monitored. LDR probes
20 matched the expected conversion products; for example, conversion of -CCGG-
template to -ACGT- required discrimination probes ending in -AC, -AT, -AG, and
-AA, and a common probe with 5' pGT-. Discrimination probes had 5' tails of
different length and a FAM label for fluorescence detection. The tail length
25 allowed physical separation of different LDR products on an acrylamide gel, and
15 thus identification of the LDR products.

LDR reactions were preincubated for 1.5 min at 94°C prior to the
30 addition of 5 nmol *Tth* ligase, followed by 10 LDR cycles of 94°C 15 sec, 65°C 2
min, and a final hold briefly at 94°C. Reactions were cold quenched and stored at
-70°C. The LDR products were separated on 10% acrylamide gels containing 7M
20 urea, with 0.6x TBE (1x TBE: 90 mM Tris base, 90 mM Borate, 2 mM EDTA) in
35 the gel and running buffer. Data were collected using an ABI 373 DNA
sequencer with Genescan 672 software.

40 Example 7 - Image Processing

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Gel pictures were produced by the ABI 672 Analysis software.
Dye specific images were opened in Adobe Photoshop 3.0, cropped, resized, and
45 converted to grayscale. The grayscale images were opened in NIH Image 1.59,
inverted, and 1D vertical background was subtracted. The background-subtracted
30 images were reinverted and rendered in pseudocolor by Photoshop to make
intensity differences easier to compare. Except for color replacement, only linear
50 image processing was performed to preserve relative intensities.

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Initial experiments were designed to determine the efficiency of generating PCR products when using probes containing 3' terminal nucleotide analogs (see above Examples). Eight different analogs were designed to pair with more than one of the four natural bases in order to convert one base to another base at a specific position in a sequence. Primer pairs containing either a nucleotide analog or one of the four natural bases at their 3' ends were used to amplify four different templates (Figure 6A). Each nucleotide analog and natural base was mispaired (or paired) in turn with all four natural bases on the opposite strand, and amplification was attempted with either *Taq* Stoeffel Fragment or *Taq* Fluorescent Sequencing polymerases. The relative amplification efficiency was determined by the number of cycles required to generate visible product on an ethidium bromide stained agarose gel, as shown in Table 1. The 50-base pair synthetic duplex DNA templates containing p53 sequence spanning codon 248 are distinguished by the four bases replacing the *MspI* site, which are shown.

Table I

TCGA template			CCGG template		GCGC template		ACGT template	
primer	reads A		reads G		reads C		reads T	
3' base	write	(eff.)	write	(eff.)	write	(eff.)	write	(eff.)
T	A	(+++)	A	(++)		(++)		(++)
C		(++)	G	(+++)		(++)		(++)
G		(++)		(++)	C	(+++)		(+++)
A		(+)		(+)	T	(+++)	T	(+++)
Q ₁	A,T	(±)		(±) ¹		(-)		(+++) ₁
Q ₂		(±) ¹		(±) ¹		(-)	T	(+++) ¹
Q ₅		(++)		(++)		(+++)	C	(+++)
Q ₆	A,G	(+++)		(+++)		(++)		(++)
Q ₇		(+)		(+)		(+++)	T	(+++)
Q ₁₆	A,T	(+) ¹		(-)		(-)		(-)
Q ₁₈		(+) ¹		(±) ¹		(±) ¹	T,A	(+++)
Q ₁₉	A	(++) ¹		(-)		(±) ¹		(±) ¹

5 1 Low product yield.

Table I. Extension efficiency and conversion with 3' natural base and nucleotide analog primers. Four different templates were used to test primer extension from a 3' base or analog paired in turn with A, G, C and T. Relative efficiency was determined by the number of cycles required to generate visible product with *Taq* Stoeffel Fragment polymerase: 10 cycles, (++) 20 cycles, (+) 30 cycles, (±) 40 to 50 cycles, (-) no product. Two of the natural base mismatch primer products were sequenced. Generally, the most efficiently amplified template for each analog was reamplified with truncated primers and sequenced to determine which bases are written opposite each analog. In one case (Q₁) a lower efficiency extension product with higher yield was selected for sequencing. Mixed base writing preference for some analogs is indicated, with most frequent product listed first.

Both *Taq* Stoeffel Fragment and *Taq* Fluorescent Sequencing

polymerases generated comparable amounts of product. Perfectly matched natural base primers generated visible product after 10 cycles, however some analog primers generated no product after 50 cycles. The analogs that did amplify with high efficiency were those that were best able to "read" the opposite strand sequence, see Figure 4.

One product for each analog (as well as the natural base controls) were reamplified and sequenced to determine polymerase preference in inserting nucleotide bases opposite the analog, shown in Table I. The Q₁, Q₅, Q₆, Q₁₆, and Q₁₈ primers generated detectable true conversion product, however only Q₅ primers generated almost exclusively true conversion product. No single analog

functioned as a "universal base" (Hill et al., "Polymerase Recognition of Synthetic Oligodeoxyribonucleotides Incorporating Degenerate Pyrimidine and Purine Bases," Proc. Natl. Acad. Sci. USA, 95(8):4258-63 (1998)) capable of generalized conversion. Unexpectedly, some products contained sequences that were difficult to read across the middle four bases, suggesting single base insertions or deletions occurred during PCR extension. This was especially prevalent in products generated from mismatched natural bases (see below).

To test the ability of convertides to reduce mismatch extension errors, the effects of preconversion PCR cycles on fidelity were assessed. PCR products generated from template amplified with only natural base conversion primers were compared to products resulting from two initial PCR cycles using convertides followed by selective amplification using specific natural base primers. Preconversion PCR was performed with primer pairs containing Q₅, Q₆, and Q₇ analogs, since these convertides had been shown to be the most efficiently extended. To improve overall PCR fidelity and 3' mismatch primer extension, CiNF buffer, buffer G(f), was used (Day et al., Nucleic Acids Res. (1999)). Nine different synthetic duplex templates containing mutated *MspI* sites were amplified with or without preconversion using 3' analog preconversion primers. Both natural base conversion primers and 3' analog preconversion primers were designed to manipulate the outside bases CCGG of the *MspI* position, shown in Figure 6A-B. Some conversions were intended to serve as controls. In these cases, the original bases in the template were either restored after analog preconversion or never changed with full-length perfect match primers. All steps were performed identically between preconversion and non-preconversion reactions, except that preconversion reactions used as template the product of 2 cycles of convertide PCR for succeeding rounds of amplification, while synthetic duplex served as the starting material for PCR with no preconversion. In both cases, 3' natural base primers were used to selectively amplify the desired endproduct. These primers contained non-hybridizing zipcode sequences on their 5' ends, which ultimately served as primer binding sites for the final 20-30 cycles of PCR, shown in Figure 6B. Conversion products were quantified by LDR, Figure 6C.

Figure 7 shows the products of the experiment. Overall, natural base mismatch conversion generated greater than 80% incorrect conversion products, shown in Figure 7A, lane 9, Figure 7B, lane 1, 3, 5, 7, 15 and 17), but preconversion could improve the fidelity and/or the yield of some conversions. In general, transversions were difficult to achieve even with preconversion. G→C and A→C conversion generated very little of the expected product with either the natural base or Q₆ primers (Figure 7A, lanes 11-14). Use of Q₆ preconversion improved the yield of G→T and A→T conversion products (compare natural base conversion in Figure 7B lanes 11, 13 with Q₆ preconversion in lanes 12 and 14). In the case of transitions, C→T conversion produced unexpected one-base shortened artifacts with natural base mismatch primers on the CXGG templates (Figure 7B, lanes 1, 3, 5, 7, 15, and 17), but the correct products were generated when using Q₆ preconversion, as seen in Figure 7B lanes 2, 4, 6, 8, 16, and 18). In addition, Q₆ primers did improve the yield of the expected T→C conversion product, shown in Figure 7A lanes 9 and 10. The controls performed as expected: all C→C and T→T nonconversion reactions worked correctly without convertides, seen in Figure 7A lanes 1, 3, 5, 7, 15, and 17, and 5B, lane 9, and the corresponding Q₆ preconversion products were restored to the original sequence, shown in Figure 7A lanes 2, 4, 6, 8, 16, and 18, and Figure 7B, lane 10). In summary, Q₆ preconversion reduced or eliminated artifacts produced by natural base C→T and T→C conversion and facilitated transitions in general. Transversions were only partially successful: G→T and A→T conversions could be improved with preconversion, but G→C and A→C conversion could not be achieved.

Apparently correct conversions were observed with attempted C→G and C→A transversions, however, carefully designed control templates revealed that these "conversions" were artifactual. C→G and C→A conversion appeared to be successful for templates containing a central CpG dinucleotide (Figure 8A and 8B, lanes 1-3, 13-21). However, the same final conversion products were observed with other templates lacking the central CpG dinucleotide, now clearly incorrect. For example, a GCGC product resulted during G conversion in reactions where the expected product should have

5 contained T, G or A in the second position (Figure 8A lanes 4-12). Also, an
ACGT product resulted during a conversion where the expected product should
10 have inserted a non-C base in the second position (Figure 8B lanes 4-12, and 22-
27). The mismatch primers used to alter the outer bases of the recognition site did
5 not reach the central dinucleotide, yet these bases were altered. It is doubtful the
"successful" conversions occurred through the intended mechanism, and thus
15 represent fortuitous artifact. The yield of LDR product was low for two
palindromic templates despite efficient PCR (lanes 22-27 in both Figure 8A and
Figure 8B). These conversion reaction products presumably contain a large
20 fraction of insertions or deletions, which cannot be detected by the current set of
LDR probes. In summary, C→G conversion was partially accomplished by both
Q₅ (Figure 8A, lanes 5, 8, 11, and 23) and the natural base G (Figure 8A, lanes 4,
7, 10, and 22); however, preconversion does not appear to improve conversion.
25 C→G conversion exhibits sequence dependence.

15 The results of the preconversion study indicate that errors in natural
base conversion were prevalent, but the use of Q₅, Q₆ and Q₇ convertides in
preconversion reduced polymerase error in certain cases. In terms of conversion
30 reactions, transitions were easier to accomplish than transversions. This is in
agreement with previous findings: Newton *et al.* observed more errors in
20 extension of primers with 3' terminal C-T, A-A, and T-T mismatches
(transversions) than with purine-pyrimidine mismatches (transitions) (Newton et
35 al., "Analysis of Any Point Mutation in DNA: The Amplification Refractory
Mutation System (ARMS)," Nucleic Acids Res. 17(7):2503-16 (1989)). Here,
pyrimidine-pyrimidine conversion usually generated the expected product,
40 especially when using convertides. In cases of purine-pyrimidine, and
pyrimidine-purine conversion, incorrect products were often generated. A
summary of results is shown in Table 2, below. Formation of incorrect
45 conversion products can be explained in part by a transient base-pair slippage of
the primer 3' nucleotide (or analog) to a misaligned position on the template, as
30 shown in Figure 9. As a result, the sequence following the mismatch is not
complementary to the original template. Consistent with this hypothesis is the
50 observation of unreadable sequence immediately following the analog in initial
sequencing experiments. Palindromic products, especially CpG dinucleotides, are

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themselves prone to slippage and extension. Palindromic products were frequently produced from non-palindromic templates. These artifacts were reduced by the presence of 10% formamide in the PCR buffer, presumably through destabilization of misaligned structures. Finally, nucleotide analogs produced fewer artifacts than natural bases. Different analogs produced different kinds and quantities of artifacts, perhaps according to their relative ability to base pair and stabilize a slippage misalignment. Thus, if polymerase extension is slow from an analog poorly base-paired with the template, extension from a strong transient base pair generated by slippage could exceed the rate of extension from a weakly base-paired 3' terminal base.

Table 2

Starting Template ^a	First base converted to			
	C	T	G	A
1 CCGG	C	Q ₆	Q ₇ (FP)	Q ₅ (FP)
2 CTGG	C	Q ₆	X (err C)	X (err C)
3 CGGG	C	Q ₆	Q ₅ (err C)	X (err C)
4 CAGG	C	Q ₆	G (err C)	X (err C)
5 TCGA	Q ₆	T or Q ₆	Q ₇ (FP)	Q ₅ (FP)
6 GCGC	X (err G)	Q ₆	G	Q ₅ or Q ₇
7 ACGT	X (err G)	Q ₆ weak	Q ₇	A or Q ₇
8 CATG	C	X	Q ₅ (err C)	X (err C)
9 CGCG	C	Q ₆	X	Q ₇ (err C)

^a The 50 bp synthetic duplex DNA templates containing p53 sequence spanning codon 248 are distinguished by the four bases replacing the *MspI* site, which are shown.

Table 2: For the most effective conversions, see Figures 5 and 6. Nine duplex DNA templates were used in conversion reactions. Each contained sequence identical to p53 surrounding codon 248, except the *MspI* site was replaced by a different four base sequence (B₁B₂B₃B₄). B₁ and B₂ (opposite strand) were simultaneously converted in turn to C, T, G and A either directly by PCR using natural base primers, or by preconversion PCR with nucleotide analog primers followed by PCR with natural base primers. In nonconversion control reactions the "conversion" product is identical to the original template. A natural base is used to indicate control reactions, and cases in which preconversion did not improve conversion. Preconversion was performed using Q₆ to facilitate conversion to C and T, and using Q₅ and Q₇ to facilitate conversions to G and A. Conversion primers determine B₁ and B₂; LDR was performed to detect unintended base changes in B₃ (which ideally is unchanged after conversion). Conversion improved by preconversion is indicated by the nucleotide analog used. Preconversion equally as effective in control reactions as natural base primers is also indicated by the analog used. Low conversion fidelity results in large B₂ error. Major B₂ error products are identified (e.g. err C indicates C at B₂), and the absence of correct product indicated no conversion method was successful (X = no correct product). Apparently correct product probably formed through a fortuitous mechanism is indicated (FP = false positive).

As discussed earlier, PCR-RFLP has been widely used to detect rare mutations. A limitation of this technique is reliance on serendipity to yield mutations that can be modified to create restriction sites in either the wild-type or the mutant template. A second limitation imposed on this approach is the need to avoid using 3' terminal mismatch primers, since extension from these primers is known to be error-prone. To date, the majority of successful attempts have used interrupted palindromic restriction sites to avoid using 3' terminal mismatch primers. Mutations in the cancer causing *K-ras* and *H-ras* were detected at a sensitivity of 1 in 10⁵ using PCR/RFLP with interrupted palindromic enzymes *XmnI* (Kumar et al., "Oncogene Detection at the Single Cell Level," *Oncogene*, 3(6):647-51

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(1988)), *AlwNI* (Anderson et al., "Prevalence of RAS Oncogene Mutation in Head and Neck Carcinomas," J. Otolaryngol., 21(5):321-6 (1992)). and *BstNI* or *MvaI* (Urban et al., "Detection of C-Ki-Ras Mutation by PCR/RFLP Analysis and Diagnosis of Pancreatic Adenocarcinomas," J. Natl. Cancer Inst., 85(24):2008-12 (1993); and Ronai et al., "Quantitative Enriched PCR (QEPCR), a Highly Sensitive Method for Detection of K-Ras Oncogene Mutation," Hum. Mutat., 10(4):322-5 (1997)). These PCR-RFLP experiments and others (Hattori et al., "Mismatch PCR RFLP Detection of DRD2 Ser311Cys Polymorphism and Schizophrenia," Biochem. Biophys. Res. Commun., 202(2):757-63 (1994); Beutler et al., "The Facile Detection of the Nt 1226 Mutation of Glucocerebrosidase by 'Mismatched' PCR," Clin. Chim. Acta., 194(2-3):161-6 (1990); Hingorani et al., "A Simple Molecular Assay for the C1166 Variant of the Angiotensin II Type 1 Receptor Gene," Biochem. Biophys. Res. Commun., 213(2):725-9 (1995); Kuwata et al., J Allergy Clin Immunol, 96(6 Pt 2):1051-60 (1995); Nishiwaki et al., "Mutational Screening of APP Gene in Patients with Early-Onset Alzheimer Disease Utilizing Mismatched PCR-RFLP," Clin. Genet., 49(3):119-23 (1996); and Ishihara et al., "Analysis of Allelic Variation of the TAP2 Gene in Sarcoidosis," Tissue Antigens, 49(2):107-10 (1997)) avoid 3' terminal mismatches, however most cancer mutations are in sequences that cannot be converted to interrupted palindromes, for example at CpG dinucleotides. A larger fraction of mutations would be made into targets for detection if contiguous recognition sequences could be introduced with as few errors as interrupted palindromic recognition sequences. Currently, contiguous restriction sites are introduced by terminal 3' mismatch primer extension, which is prone to errors. O'Dell et al. tested a general method for introducing different restriction sites at CpG dinucleotides using mismatch PCR (O'Dell et al., "PCR Induction of a *TaqI* Restriction Site at Any CpG Dinucleotide Using Two Mismatched Primers (CpG-PCR)," Genome Res., 6(6):558-68 (1996)). The outer bases of four different CpG dinucleotides in the human LDL receptor gene were altered to create *TaqI* (TCGA), *MspI* (CCGG) or *RhaI* (GCGC) sites. In these targets, *TaqI* sites were successfully generated by 3' T mismatch primers. The method was able to detect homozygous and heterozygous individuals; however, the ratio of products representing each allele was not equal, as is expected in germline

5 mutations. Several cases have been shown here where T mismatch conversion
failed to create a *TaqI* site. Thus the method is sequence dependent. O'Dell et al.
10 found that C and G mismatch conversion failed. These results agree with
O'Dell's conclusion that stronger base pairing leads to mispriming, possibly
5 through stabilization of primer slippage on the template. Gotoda et al., "Detection
of Three Separate DNA Polymorphisms in the Human Lipoprotein Lipase Gene
15 by Gene Amplification and Restriction Endonuclease Digestion," J. Lipid Res.,
33(7):1067-72 (1992), claim to have successfully used PCR-RFLP to introduce a
MaeII site (ACGT) by extension of a 3' C-A mismatch to produce a T→C
20 transition (Gotoda et al., "Detection of Three Separate DNA Polymorphisms in
the Human Lipoprotein Lipase Gene by Gene Amplification and Restriction
Endonuclease Digestion," J. Lipid Res., 33(7):1067-72 (1992)). Athma et al. used
PCR extension of a 3' terminal mismatch primer to create a restriction site for
25 discriminating between two alleles (Athma et al., "Single Base Polymorphism
Linked to the Ataxia-Telangiectasia Locus is Detected by Mismatch PCR,"
15 Biochem. Biophys. Res. Commun., 210(3):982-6 (1995)). A G-T mismatch
produced an *MvaI* site (CC A/T GG) through an A→G transition. Successful
A→G conversions have been performed in accordance with the method of the
present invention, using a natural base mismatch, but difficulties with T→C
20 conversion by natural base primers has been encountered. Transitions can be
accomplished more readily than transversions, but the yield of correct product can
35 be sequence dependent. Others have also found that PCR-RFLP can produce
false-positive results (Hodanova et al., "Incorrect Assignment of N370S Mutation
Status by Mismatched PCR/RFLP Method in Two Gaucher Patients," J. Inherit.
40 Metab. Dis., 20(4):611-2 (1997)). The use herein of ligase detection reaction
25 allows the determination of the precise amounts of misextension products
generated.

45 The fidelity of polymerase extension from primers containing 3'
natural bases and nucleotide analogs has been measured. Results indicate that
30 natural base mismatch primer extension cannot be used as a general technique to
create restriction sites in any given sequence for RFLP analysis. Primer slippage
50 appears to be an important mechanism for producing error in mismatch primer

extension. This source of error may have a dramatic impact on some allele-specific PCR and other methods of high sensitivity mutation detection. With further development and testing of nucleotide analogs to facilitate conversion, mismatch primer extension may become a technique that can efficiently introduce desired mutations with few artifacts. It has been found some nucleotide analogs improve mismatch primer extension (see Table 3, below). Further improvement of 3' mismatch extension will be required to minimize the high degree of context dependent error seen in transversions and lead to improved levels of sensitivity and broader scope of PCR/RFLP based mutation detection.

Table 3

Starting Base	Conversion to			
	C	T	G	A
C	C	Q ₆		
T	Q ₆	T		
G			G	A or Q ₇
A			Q ₅ or Q ₇	A

Table 3. Summary of conversion strategy. A Q_n convertide indicates preconversion is required using the indicated convertide prior to final conversion using natural base primers. In some cases, an additional convertide or using only the natural base will result in the desired conversion.

Example 9 - Oligonucleotide Synthesis for PCR/RE/LDR

Oligonucleotides were synthesized at the 0.2 μ mole scale by cyanoethyl phosphoramidite chemistry on an Applied Biosystems 394 DNA synthesizer. Standard 500Å CPG columns and reagents (Applied Biosystems) were used with the following exceptions: oligonucleotides 50 bases in length were synthesized using wide-pore 1000Å CPG columns (Applied Biosystems); oligonucleotides with fluorescent dye FAM at the 5' terminus were synthesized using FAM phosphoramidite (Applied Biosystems) with a 15 minute coupling step; oligonucleotides with 5' phosphate were synthesized using phosphorylation reagent (Glen Research) with a 15 minute coupling step. Oligonucleotides with 3' blocking group were synthesized using 3'-Spacer CPG columns (Glen Research).

Oligonucleotides with the 3' nucleotide analog 6-(2-deoxy-B-D-ribofuranosyl)-6H,8H-3,4-dihydropyrimido[4,5-c][1,2]oxazine-7-one (Q₆) were synthesized using dP-CpG (Glen Research).

5 Example 10 - PCR Polymerases and Buffers for PCR/RE/LDR

The polymerases used were *AmpliTaq* (Applied Biosystems), *Vent* and *Vent(exo-)* (New England Biolabs), and Expand polymerase mix (*Taq* and *PwoI* polymerase mixture, in Expand High Fidelity kit, Boehringer Mannheim).

The commercially available PCR buffers used were supplied in the *AmpliTaq* and Expand kits. Tris pH 9.1 (pH values were measured using 1 M stock solutions at 23°C), tricine pH 8.7, EPPS (N-[2-hydroxyethyl]piperazine-N'-3-propanesulfonic acid) pH 8.4, and citrate pH 7.6 (Sigma) were used for alternative PCR buffers.

Unless otherwise noted, each 20 µl reaction contained 20 mM Tris, tricine, or citrate, 200 µg/ml bovine serum albumin, 2.5 mM MgCl₂, 200 µM dNTP (each) and either 16 mM (NH₄)₂SO₄, or 50 mM potassium acetate. Formamide at 10% concentration was used as indicated (see Example 11, below). PCR buffers were made as 10% stocks requiring the addition of formamide as needed, dNTPs, and the oligonucleotide primers and template DNA.

10 Example 11 - Enzyme Buffer Notation

Test PCR buffers are named to indicate the presence of one or more components: Tris/potassium acetate = buffer A; Tris/ammonium sulfate = buffer B; Tricine/ammonium sulfate = buffer D; EPPS/potassium sulfate = buffer E; EPPS/ammonium sulfate = buffer F; and Citrate/ammonium sulfate = buffer G. Component concentrations are described above.

15 Example 12 - Amplification of p53 exon 7 from Genomic DNA

Part of p53 exon 7 surrounding codon 248 was amplified as shown in Figure 10. The upstream primer has nucleotide sequence corresponding to

5 SEQ. ID. NO. 1, as follows:

5'-GCCTCATCTTGGGCCTGTGTTATC-3'

10 hybridized within the preceding intron, and the downstream primer, with a
5 nucleotide sequence corresponding to SEQ. ID. NO. 2, as follows:

15 5'-GTGGATGGGTAGTAGTATGGAAGAAATC-3'

20 hybridized within exon 7. All PCR, restriction endonuclease digestion and
ligation steps described throughout were performed using a GeneAmp PCR
System 2400 (Perkin-Elmer). Several buffers and enzymes were used as indicated
in Examples 10 and 11. The p53 exon 7 amplification from genomic DNA was
performed starting with a 20 μ l reaction mixture containing 50 ng of DNA, 2.5
mM of each dNTP, and 12.5 pmol of each primer in 1x buffer without polymerase.
25 The reaction mixture was covered with paraffin oil and preincubated for at least
1.5 min at 94°C in order to perform hot start by adding 1 μ l of polymerase diluted
in 1x buffer to introduce the required units of polymerase. The exon 7 segment
was amplified for 40 cycles of 94°C 15 sec, 65°C 2 min, with an additional 5 min
at 65°C at the end of the last cycle. PCR amplifications departing from this
30 procedure were performed as indicated.

35 **Example 13 - PCR/RE/LDR: Fidelity Assay**

40 Templates were amplified with conversion primer pairs bracketing
the central two basepairs of the *MspI* site (CCGG) at codon 248 (Figure 11B).
25 Tubes were prepared containing 10 fmol per reaction of either PCR amplified p53
exon 7 or wild-type synthetic duplex template, PCR buffer, and primers. In
parallel reactions, a synthetic 50 bp duplex marker template (MK), with the
45 sequence CGGG replacing the *MspI* site at codon 248, was added at 10^{-3} , 10^{-4} ,
30 10^{-5} and 0 molar ratio to wild-type template. Reactions were preincubated for at
least 1.5 min at 94°C with all components present in CiNF buffer, buffer G(f),
50 except Vent (exo-) polymerase. A "hot start" was performed by adding 1 μ l of

polymerase at 94°C. When preconversion was performed, 2 cycles of 94°C 15 sec, 55°C 1 min, 60°C 1 min were executed with 500 fmol each of the primers p53-248Q₆ and p53-248Q₆R. Afterwards, 1 pmol of p53Taq248T and p53Taq248TR primers were added. When preconversion was not performed, the reactions contained 1 pmol each of the primers p53Taq248T and p53Taq248TR or the control primers p53Msp248C and p53Msp248CR. After reactions with and without preconversion were performed, conversion PCR was carried out as follows: 5 cycles of 94°C 15 sec, 55°C + 1°/cyc 1 min (temperature ramp), 60°C 1 min; then, 20 cycles of 94°C 15 sec, 60°C 2 min; then, a final 60°C 5 min extension. After 3 cycles of the temperature ramp 10 pmol of long zipcode conversion primers (p53zip248T and p53zip248TR, or p53zip248C and p53zip248CR) were added. After conversion, the wild-type DNA was digested periodically during 20 cycles of "zipcode" PCR (described below). Polymerase was inactivated by freezing and thawing twice. Finally, LDR was performed to detect the conversion products without contribution from the original template (except in nonconversion control reactions).

Example 14 - PCR/RE/LDR: "Zipcode" PCR

Wild-type sequences or wild-type conversion products were removed by restriction digestion. The appropriate restriction endonuclease was added to the reaction tube and supplemented with additional MgCl₂ as required to allow efficient digestion. *MspI* digestion was performed at 37°C for 15 min using no additional MgCl₂, except when using citrate buffer. *TaqI* digestion was performed at 65°C for 30 min at 6 mM Mg²⁺ by adding 1 µl of enzyme diluted in 140 mM MgCl₂. The undigested conversion products were reamplified from 1 µl of a 10x dilution added to a 20 µl PCR reaction containing 10 pmol of the "zipcode" primers Ztop and Zbot (Figure 6B). These zipcode primers each contain a DNA sequence that is not similar in sequence to any genomic sequences present in the sample, thus only the products of previous PCR using primers containing the zipcode sequences will be efficiently amplified. Conversion products were amplified using Expand polymerase mix and buffer (see Example

- 5 10) After an initial RE digest, zipcode PCR reamplification followed by
redigestion was performed as follows: reactions were preincubated at 94°C for at
10 least 1.5 min then initiated with a hot start by adding 0.1 µl of RE digested sample
(1 µl of a 10x dilution) to a 20 µl reaction; 10 cycles of 94°C 15 sec, 65°C 2 min.
5 Zipcode PCR amplification products were redigested as described above.

15 **Example 15 - PCR/RE/LDR: Ligase Detection Reaction**

- Ligase detection reactions were performed in standard LDR buffer
20 (25 mM Tris pH 7.6, 12 mM MgCl₂, 65 µg/ml bovine serum albumin, 100 mM
KCl, and 10 mM DTT). Each 20 µl reaction contained approximately 500 fmol of
dsDNA (1 µl of PCR sample), 500 fmol of each discriminating probe, and 750
25 fmol of common probe, shown in Figure 11C. Sets of discrimination and
common probes were synthesized to detect the expected conversion products; *i.e.*,
15 converted to CNGG or TNGA at the *MspI* position. The common probe was
synthesized using 3'-Spacer C3 CPG columns and the 5' end was phosphorylated
on the column using phosphorylation reagent. Discrimination probes of each set
30 varied at the 3' terminal base to query the base in that location, *i.e.*, the second
base of the *MspI* position. Discrimination probes had 5' tails of different length
20 and a FAM label for fluorescence detection. The tail size identified the probe and
allowed physical separation of different LDR products on an acrylamide gel.

- The LDR reaction was preincubated for 1.5 min at 94°C prior to
the addition of 5 nmol *Tth* ligase enzyme under a layer of mineral oil. 10 LDR
40 cycles of 94°C 15 sec, 65°C 2 min. were used. The reactions were then held at
25 94°C until cold quenched on ice and stored at -70°C. The LDR products were
separated on 10% acrylamide gels containing 7M urea with 0.6x TBE (1x TBE
contains 90 mM Tris base, 90 mM Borate, 2 mM EDTA) used in the gel and for
45 the running buffer. Data were collected using an ABI 373 automated DNA
sequencer and Applied Biosystems Genescan 672 software (GS Collection and GS
30 Analysis).

Example 16 - Image Processing for PCR/RE/LDR

Raw gel pictures were produced by the ABI GS Analysis software. Dye specific pictures were opened in Adobe Photoshop 3.0, cropped, resized, and converted to grayscale. The grayscale images were opened in NIH Image 1.59, inverted and 1D vertical background was subtracted. Optionally, NIH Image could render a 3-D plot from a corrected 2-D picture. Background corrected pictures were reinverted and rendered in pseudocolor by Photoshop by replacing the color table to make subtle intensity differences easier to compare. Except for color replacement, only linear image processing was performed in order to preserve relative intensities.

Example 17 -PCR/RE/LDR Optimization

In Figure 10, PCR/RE/LDR was developed to detect and identify low abundance mutations occurring within the *MspI* site (CCGG) at codon 248 in the p53 gene. An initial PCR amplifies exon 7 from genomic DNA, shown in Figure 10A. This product serves as the template for a second PCR that amplifies the central CpG dinucleotide in the *MspI* site. To generate a restriction site in sequence lacking a preexisting site, mismatch primers are used to alter one or more bases flanking the CpG dinucleotide. This results in a conversion PCR that creates a restriction site (NCGN → TCGA) *TaqI* site, for example, as in Figure 10B. In a generalized method for introducing contiguous Type II restriction sites, conversion PCR primers by necessity have 3' terminal mismatches. To avoid unfavorable natural base mismatches that may result in insertion of an erroneous base at the next site (O'Dell et al., Genome Res., 6(6):558-68 (1996); and Eiken et al., "Application of Natural and Amplification Created Restriction Sites for the Diagnosis of PKU Mutations," Nucleic Acids Res., 19(7):1427-30 (1991)), preconversion with 3' nucleotide analog primers is performed. However, extension with 3' analog primers produces a pool of degenerate products (Day et al., Nucleic Acids Res., (1999)). Thus, after this preconversion step, natural base primers are used to selectively amplify the desired products.

5 Mismatch conversion error relative to PCR error was assessed by
performing parallel nonconversion control reactions and true conversion reactions
10 with and without preconversion. Nonconversion reaction products retained the
MspI site (CCGG) (see Figure 10A), while conversion introduces a *TaqI* site
5 (TCGA) (see Figure 10B). All PCR/RE/LDR steps were performed under similar
conditions, varying only the primers and restriction endonuclease (*MspI* or *TaqI*).
15 In both cases, noncleavable DNA is preferentially amplified. When wild-type
DNA is selectively removed by digestion, it is necessary to determine the
proportion of DNA with incorrect sequence produced relative to the initial
20 quantity of DNA in the sample, which is nearly 100% wild-type. Parallel
reactions were performed in which known fractions of marker (MK) DNA were
present. The MK DNA contained a single base change in the *MspI* site (CGGG),
rendering it uncleavable by *MspI*. C→G transversions are unlikely to occur
25 through polymerase error. The MK standard curve allows quantification of
mutations detected by LDR. Figure 10C shows the LDR quantification of mutant
sequencing using a marker. PCR conditions were tested to minimize PCR error
(observed in the nonconversion reactions) and mismatch extension errors
30 (additional errors observed in the conversion reactions).

Various proofreading and nonproofreading polymerases were
20 tested, as different polymerase properties are required during target amplification
from genomic DNA, conversion, and reamplification steps in PCR/RE/LDR.
35 Since it is essential throughout PCR/RE/LDR to minimize any alteration of the
bases assayed by LDR, proofreading polymerases might seem the logical choice
for maintaining the highest fidelity (Keohavong et al., PCR Meth. Appl., 2:288-92
40 (1993)); however, they may interfere with conversion by mismatch primer
extension. Hence, PCR conditions must be found which maximize the fidelity of
nonproofreading polymerases (Keohavong et al., Proc. Natl. Acad. Sci. USA,
45 86(23):9253-7 (1989)).

Initially, PCR/RE/LDR was used as a high sensitivity assay to
30 determine PCR conditions that maintain the highest fidelity throughout the
procedure. Two main sources of error were expected: 1) polymerase
50 misincorporation, and 2) DNA template degradation. Raising the PCR buffer pH
improves long PCR, probably by decreasing depurination which leads to strand

5 cleavage (Barnes, "PCR Amplification of up to 35-Kb DNA with High Fidelity
and High Yield from Lambda Bacteriophage Templates," Proc. Natl. Acad. Sci.
10 USA, 91(6):2216-20 (1994); Cheng et al., "Effective Amplification of Long
Targets from Cloned Inserts and Human Genomic DNA," Proc. Natl. Acad. Sci.
5 USA, 91(12):5695-9 (1994); and Sang et al., "Generation of Site-Directed
Mutagenesis by Extralong, High-Fidelity Polymerase Chain Reaction," Anal.
15 Biochem., 233(1):142-4 (1996)). While higher pH may decrease template
damage, higher pH is also known to adversely affect polymerase fidelity (Eckert
et al., "High Fidelity DNA Synthesis by the *Thermus Aquaticus* DNA
20 Polymerase," Nucleic Acids Res., 18(13):3739-44 (1990); Eckert et al., "DNA
Polymerase Fidelity and the Polymerase Chain Reaction," PCR Methods Appl.,
1(1):17-24 (1991); and Cline et al., "PCR Fidelity of Pfu DNA Polymerase and
25 Other Thermostable DNA Polymerases," Nucleic Acids Res., 24(18):3546-51
(1996)). Therefore, other buffers were also tested: tricine, EPPS, and citrate
15 buffers, which have pK_a s in the range of 7 to 8 and $|\Delta pK_a|$ lower than Tris. Tris
cannot meet the dual constraints of a slightly alkaline pH at high temperature to
maintain template integrity and neutral pH at the extension temperature to
30 maintain polymerase fidelity, although most PCR fidelity and long PCR studies
use Tris. Some investigators have explored the use of alternative buffers with
20 lower $|\Delta pK_a|$ (Eckert et al., "High Fidelity DNA Synthesis by the *Thermus*
Aquaticus DNA Polymerase," Nucleic Acids Res., 18(13):3739-44 (1990); Eckert
et al., "DNA Polymerase Fidelity and the Polymerase Chain Reaction," PCR
35 Methods Appl., 1(1):17-24 (1991); and Brail et al., "Improved Polymerase
Fidelity in PCR-SSCPA," Mutat. Res., 303(4):171-5 (1993)). Buffer-specific
40 effects on PCR were tested for purposes of optimizing the buffer conditions of the
present invention by making PCR buffers containing identical components except
for the buffering compound. Making one set of test PCR buffers with ammonium
45 sulfate and another with potassium acetate tested salt effects. The ΔpK_a of each
buffer was determined in pure solution and in 1x PCR buffer mixtures (data not
30 shown). Results of these tests agreed with published ΔpK_a values of pure buffers
(Good, "Amine Buffers Useful for Biological Research," in Fasman, ed.,
50 Handbook of Biochemistry and Molecular Biology, Cleveland, Ohio: CRC Press,

Inc., pp. 367-369 (1976); and Blanchard, "Buffers for Enzymes," *Meth. Enzymol.*, 104:404-14 (1984)) corrected by a small constant (.005 pH units/°C), possibly due to a temperature-dependence of the pH probe itself. The pH of the each test PCR buffer was adjusted to produce approximately neutral pH at 65°C. However, the 1x PCR buffers had somewhat different ΔpK_a s compared to the pure buffers; for example, 1x TsN, buffer B, had $\Delta pK_a = -.033/^\circ\text{C}$ versus $-.030/^\circ\text{C}$ for 100 mM Tris, and 1x TcK, buffer C, had $\Delta pK_a = -.022/^\circ\text{C}$ versus $-.025/^\circ\text{C}$ for 100 mM tricine.

Test PCR buffers containing Tris, tricine, or EPPS were used to test PCR fidelity with no conversion of the *MspI* site (CCGG) at codon 248 of p53 (Figure 12). The objective in this experiment was to test the error rate of PCR using various buffers and polymerase enzymes. Since introduced errors create template that cannot be cleaved by the selected restriction enzyme, false-positives accumulate as this error template continues to amplify alongside true mutant DNA. This established the conditions necessary to achieve amplification while minimizing error. The same polymerase and buffer set was used in both preamplification of p53 exon 7 from genomic DNA and in the "conversion" step. As mentioned, the "conversion" step maintains the *MspI* site by using perfectly matched primers whose 3' ends terminate on the C and G bases flanking the central CpG. After an initial *MspI* digest, template and amplification products were periodically redigested every ten cycles during reamplification to remove WT sequence. Synthetic marker mutant MK with the sequence CGGG was present in these reactions at 10^{-3} , 10^{-4} , or 10^{-5} and 0 ratio to wild-type (WT). MK will not be cleaved by *MspI* restriction digestion, but will amplify with each PCR cycle to provide an internal control to measure product quantities (see below). The MK product will also maintain its sequence, as the perfect match primers in the conversion step will again terminate on the C and G bases flanking the central GG. Error products resulting from MK PCR will in general lack *MspI* sites and will be indistinguishable from regular MK template. If an *MspI* site is accidentally created, the product will be destroyed by digestion. If false LDR error products are also generate, these can be detected by comparison with a "no marker" control.

For each buffer, LDR detected MK products in each of the four parallel reactions, with the 0 MK control indicating the background level of C₃GGG error produced. The intensities of other error products detected by LDR were compared to MK to estimate the fraction of each error product generated. AmpliTaq generated few transversions (C→G or C→A), but a large amount of C→T transition was observed, as shown in Figure 12A. Vent generated much less of the C→T transition compared to AmpliTaq (Figure 12B). AmpliTaq showed little dependence on the presence of potassium acetate in buffers A, C, and E (Figure 12A lanes 1-4, 9-12, 17-20) versus ammonium sulfate in buffers B, D, and F (Figure 12A lanes 5-8, 13-16, 21-24). Vent polymerase amplified template more efficiently in Tris/ammonium sulfate buffer B than Tris/potassium acetate buffer A (Figure 12B lanes 1-4 versus lanes 5-8), as described previously (Barnes, "PCR Amplification of up to 35-Kb DNA with High Fidelity and High Yield from Lambda Bacteriophage Templates," *Proc. Natl. Acad. Sci. USA*, 91(6):2216-20 (1994); Keohavong et al., *PCR Meth. Appl.*, 2:288-92 (1993); Cariello et al., "Fidelity of Thermococcus Litoralis DNA Polymerase (Vent) in PCR Determined by Denaturing Gradient Gel Electrophoresis," *Nucleic Acids Res.*, 19(15):4193-8 (1991); and Mattila et al., "Fidelity of DNA Synthesis by the Thermococcus Litoralis DNA Polymerase--An Extremely Heat Stable Enzyme with Proofreading Activity," *Nucleic Acids Res.*, 19(18):4967-73 (1991)). However, Vent exhibited improved fidelity in tricine/potassium acetate buffer C (Figure 12B lanes 9-12) and EPPS buffer E (Figure 12B lanes 17-20) compared to tricine/ammonium sulfate buffer D (Figure 12B lanes 13-16) and EPPS/ammonium sulfate buffer F (Figure 12B lanes 21-24).

The relative fidelities of the different polymerase-buffer combinations may be described by their "sensitivity" expressed as the $-\log_{10}$ of the ratio of MK to WT initially present. The C→T error for AmpliTaq amplification in Tris/potassium acetate buffer A can be taken as an example. If the signal for the CTGG error product (Figure 12A lane 2) is compared to the MK C₃GGG signal (Figure 12A lanes 1-3), the intensity of the signal most resembles the 10^{-3} MK:WT dilution (Figure 12A lane 1). Thus, the C→T error rate is 10^{-3} ; the sensitivity is 3, since $-\log[\text{MK}/\text{WT}] = -\log[10^{-3}] = 3$. From this it can be seen

that the higher the sensitivity, the lower the error rate. Reactions with higher sensitivities for each mutation had the highest overall fidelity (Results summarized in Table 1). Many of the Vent reactions had sensitivities of 5 (i.e., 1 in 10^5) for every mutation (Figure 12B), while the AmpliTaq reactions had sensitivities of 3 (i.e., 1 in 10^3), as shown in Figure 12A. Sensitivity indicates the usefulness of the assay rather than the error rate of the polymerase. Error (ER) per base per cycle may be estimated from the fraction (F) of all mutations occurring at one base which accumulated over 65 cycles (D) before digestion. For purposes of the present invention, the number of cycles is an estimate of the number of duplications, since multiple non-saturating PCRs were performed. From $ER=F/D$, Vent polymerase had an error rate of less than 1×10^{-7} /base/cycle in tricine/potassium acetate buffer C, approximately 2×10^{-7} /base/cycle in tricine/ammonium sulfate buffer D, and 2×10^{-6} /base/cycle in TsN, buffer B. An error rate of 2×10^{-5} /base/cycle was observed. This was due mainly to the C→T transition for AmpliTaq in tris/potassium acetate buffer A. Elimination of this artifact could improve AmpliTaq fidelity by more than 10-fold. Others have used cloning and screening methods to estimate polymerase error (Eckert et al., "High Fidelity DNA Synthesis by the Thermus Aquaticus DNA Polymerase," Nucleic Acids Res., 18(13):3739-44 (1990); Cline et al., "PCR Fidelity of Pfu DNA Polymerase and Other Thermostable DNA Polymerases," Nucleic Acids Res., 24(18):3546-51 (1996); Mattila et al., "Fidelity of DNA Synthesis by the Thermococcus Litoralis DNA Polymerase--An Extremely Heat Stable Enzyme with Proofreading Activity," Nucleic Acids Res., 19(18):4967-73 (1991); and Huang et al., "Fidelity and Predominant Mutations Produced by Deep Vent Wild-Type and Exonuclease-Deficient DNA Polymerases During in Vitro DNA Amplification," DNA Cell Biol., 15(7):589-94 (1996)), and denaturing gradient gel electrophoresis (DGGE) has also been used (Keohavong et al., PCR Meth. Appl., 2:288-92 (1993); Keohavong et al., Proc. Natl. Acad. Sci. USA, 86(23):9253-7 (1989); Cariello et al., "Fidelity of Thermococcus Litoralis DNA Polymerase (Vent) in PCR Determined by Denaturing Gradient Gel Electrophoresis," Nucleic Acids Res., 19(15):4193-8 (1991); and Ling et al., "Optimization of the Polymerase Chain Reaction with Regard to Fidelity: Modified T7, Taq, and Vent DNA Polymerases," PCR Methods Appl., 1(1):63-9

(1991)). However, these methods do not directly measure mutated DNA, and do not detect all mutations. By cloning and DGGE methods, Vent polymerase has an error rate estimated from 0.3 to 4×10^{-5} /base/cycle (Caricillo et al., "Fidelity of Thermococcus Litoralis DNA Polymerase (Vent) in PCR Determined by Denaturing Gradient Gel Electrophoresis," Nucleic Acids Res., 19(15):4193-8 (1991); and Ling et al., "Optimization of the Polymerase Chain Reaction with Regard to Fidelity: Modified T7, Taq, and Vent DNA Polymerases," PCR Methods Appl., 1(1):63-9 (1991)). The error rate of Taq polymerase has been estimated from 0.8 to 9×10^{-5} /base/cycle (Eckert et al., "High Fidelity DNA Synthesis by the Thermus Aquaticus DNA Polymerase," Nucleic Acids Res., 18(13):3739-44 (1990); Cline et al., "PCR Fidelity of Pfu DNA Polymerase and Other Thermostable DNA Polymerases," Nucleic Acids Res., 24(18):3546-51 (1996); and Brail et al., "Improved Polymerase Fidelity in PCR-SSCPA," Mutat. Res., 303(4):171-5 (1993)), comparable to the error rate we observed for AmpliTaq in Tsk, buffer A. Of the thermostable polymerases, Pfu has the lowest reported error rate estimated from 0.7 to 1.6×10^{-6} /base/cycle (Cline et al., "PCR Fidelity of Pfu DNA Polymerase and Other Thermostable DNA Polymerases," Nucleic Acids Res., 24(18):3546-51 (1996); Lundberg et al., "High-Fidelity Amplification Using a Thermostable DNA Polymerase Isolated from Pyrococcus Furiosus," Gene, 108(1):1-6 (1991); and Andre et al., "Fidelity and Mutational Spectrum of Pfu DNA Polymerase on a Human Mitochondrial DNA Sequence," Genome Res., 7(8):843-52 (1997)). Pfu polymerase may also exhibit improved fidelity in tricine or other low $[\Delta pK_a]$ buffers.

While high fidelity proofreading enzymes appeared to improve amplification from genomic DNA, proofreading still must be avoided in the conversion step. Different high fidelity genomic amplification conditions were tested in combination with fixed conversion conditions. Genomic amplification was performed with either Vent(exo-) Citrate/ammonium sulfate, buffer G, or Vent(exo-) Citrate/ammonium sulfate, buffer G, with 10% formamide buffer G(f) (see Table 4). Nonconversion primers were used with Vent(exo-) to optimize PCR fidelity in anticipation of conversion by mismatch primer extension. The highest fidelity conditions were as follows: Genomic amplifications with Vent/ Buffer G were initiated by spiking genomic amplification product from Expand/

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Buffer C with 10% formamide reactions after 3 cycles. These Vent/ Buffer G reactions required 4 mM Mg^{2+} and PCR primers, but no additional genomic DNA was provided. See Table 4 for observed error rate with other conditions tested.

Table 4

Polymcrase enzymes 1) Genomic DNA PCR → 2) conversion	Buffer 1)→2)	Limiting error	Error rate total	per cycle ^a
<i>Taq</i> → <i>Taq</i>	A→A	C→T	10 ⁻³	2x10 ⁻³
"	B→B	"	10 ⁻³	2x10 ⁻³
"	C→C	"	10 ⁻³	2x10 ⁻³
"	D→D	"	10 ⁻³	2x10 ⁻³
"	E→E	"	10 ⁻³	2x10 ⁻³
"	F→F	"	10 ⁻³	2x10 ⁻³
<i>Taq</i> ^b → Vent	A→A	C→T	>10 ⁻³	>2x10 ⁻³
Vent → Vent	B→B	"	10 ⁻⁵	2x10 ⁻⁷
"	C→C	"	>10 ⁻⁵	>2x10 ⁻⁷
"	D→D	"	10 ⁻¹	2x10 ⁻⁶
"	E→E	"	>10 ⁻⁵	>2x10 ⁻⁷
"	F→F	"	10 ⁻⁵	2x10 ⁻⁷
Vent (exo-) →	C→G	C→T	10 ⁻¹	2x10 ⁻⁶
Vent (exo-) → Vent (exo-)	C→G	"	10 ⁻³	2x10 ⁻⁵
Vent → Vent (exo-)	C→G	"	10 ⁻⁴	2x10 ⁻⁶
Vent → Vent (exo-)	C→G(f)	"	10 ⁻³	2x10 ⁻⁷
Vent ^c → Vent (exo-)	G(4)→G	"	10 ⁻⁴	2x10 ⁻⁶
Vent → Vent (exo-)	G(8)→G(f)	"	10 ⁻⁵	2x10 ⁻⁷
Vent ^c → Vent (exo-)	G(4)→G(f)	"	>10 ⁻⁵	>2x10 ⁻⁷

^a Based on minimum of 50 total cycles, i.e. observed error +50.

^b No vent PCR product from genomic DNA. Used *Taq* amplified product for Vent conversion PCR.

^c Template added by taking 1 µl after 3rd PCR cycle from a parallel genomic DNA amplification using Expand polymerase mix in Buffer C.

Table 4. Comparison of fidelity using proofreading and nonproofreading polymerases in different buffers for PCR to amplify the target sequence from genomic DNA and for conversion PCR. *Taq* and Vent polymerases were initially tested using one buffer for genomic amplification and conversion. During the conversion step, only nonconversion of the *MspI* site near p53 codon 248 was performed using short perfect match primers (Figure 11A) to determine the background level of polymerase error. LDR quantified *MspI* site mutations at the second position (CCGG → CNGG). Fidelity was compared in parallel reactions using proofreading and nonproofreading polymerases in genomic amplification and conversion. Expand polymerase mix. *Taq* with proofreading *Pfu* polymerase added, was used to initiate target amplification from genomic DNA for subsequent high fidelity Vent polymerase PCR. Vent polymerase was substituted with nonproofreading Vent(exo-) to determine whether proofreading was required and also in the conversion step where proofreading is not permitted. The effect of 10% formamide in the conversion PCR buffer was also tested. All buffers contained 200 µg/ml bovine serum albumin, 2.5 mM MgCl₂, and 200 µM dNTP (each). Specific components were: A (TsK): 20 mM Tris pH 9.1, 50 mM potassium acetate (standard *Taq* polymerase buffer). B (TsN): 20 mM Tris pH 9.1, 16 mM ammonium sulfate (standard Vent polymerase buffer). C (TeK): 20 mM tricine pH 8.7, 50 mM potassium acetate. D (TeN): 20 mM tricine pH 8.7, 16 mM ammonium sulfate. E (EpK): 20 mM EPPS pH 8.4, 50 mM potassium acetate. F (EpN): 20 mM EPPS pH 8.4, 16 mM ammonium sulfate. G (CiN): 20 mM citrate pH 7.6, 16 mM ammonium sulfate. (f) indicates presence of 10% formamide. (4) indicates increase to 4 mM MgCl₂. (8) indicates increase to 8 mM MgCl₂.

PCR conditions were found for each step in PCR/RE/LDR that maintain high fidelity when no mismatch conversion was performed. Using known high fidelity PCR conditions, conversion was also tested by changing the p53 codon 248 *MspI* site (CCGG) into a *TaqI* site (TCCA). MK (CGGG) was

5 added as before in parallel reactions to measure fidelity relative to the initial wild-
type DNA present. High fidelity PCR was performed as described above and
10 some (but not all) reactions were subjected to preconversion. Preconversion was
performed using primers containing the degenerate pyrimidine nucleotide analog
5 Q₆ at their 3' ends, as shown in Figure 10B. The final conversion was
accomplished using natural base 3' T mismatch primers. Products were detected
15 using LDR to interrogate the second base position in the *MspI*, *TaqI* and MK
sequence: CNGG or TNGA. Fidelity for conversion with and without
preconversion was compared to a nonconversion control. Successful conversion
20 will change the *MspI* site (CCGG) into a *TaqI* site (TCGA); MK will also be
converted from CGGG to TGGA. However, the main issue of conversion success
is the maintenance of the central bases in all cases: CpG for *TaqI* conversions and
GpG for MK. Figure 13 shows the results of conversion. In Figure 13, lanes 1-4
25 (C:G) are nonconverted reactions that were digested with *MspI*; lanes 5-8 (Q₆:G)
are preconverted/converted reactions that were digested with *TaqI*; lanes 9-12
(T:G) are converted reactions lacking preconversion that were digested with *TaqI*.
PCR/RE/LDR with no conversion was sensitive to better than 1 in 10⁴ using the
30 previously determined best conditions for preamplification and conversion shown
in Figure 13, C:G lanes 1-4. PCR/RE/LDR with conversion of the *MspI* site to a
20 *TaqI* site by T mismatch primers was apparently very successful at first glance,
Figure 13, T:G lanes 9-12. As would be expected for successful conversion, no
35 *MspI* product can be detected in the _CG_ region of the figure; hence, it appears
that the site was converted to *TaqI* and then digested. However, although a very
large fraction of MK (CGGG) is observed in the reactions with added MK,
40 Figure 13, T:G lanes 9-11; the same large fraction is also observed in the 0 MK
control lane in Figure 13, T:G lane 12. Thus, the entire quantity of MK is an
artifact produced by mismatch extension of the natural base T primers. This event
45 would convert the second position C in the *MspI* site to a G during extension,
mimicking the internal sequence of MK (CCGG→TGGA). Preconversion with
30 Q₆ primers eliminates the MK artifact, Q₆:G lanes 5-8.

50 The greater amount of WT present in nonconverted samples,
shown in Figure 13 lanes 1-4, compared to Q₆ converted samples, lanes 5-8, may
be due to inhibition of *MspI* digestion by formamide. Formamide apparently

inhibits *MspI* digestion as evidenced by the presence of strong wild-type LDR bands (WT) in the nonconversion control, C:G lanes, which are not present after *TaqI* digestion of the converted sequence, Q₆:G and T:G lanes.

The low amount of MK product seen in the Q₆ 10⁻⁴ and 10⁻⁵ MK lanes (Figure 13, Q₆:G lanes 6, 7) compared to the respective nonconversion control reactions, (Figure 13, C:G lanes 2, 3) may be due to low efficiency of MK conversion. The production of a *TaqI* site actually requires two conversions, one on each side of the central CpG dinucleotide. Lowering the concentration of MK 10-fold may reduce MK conversion far more than 10-fold. Thus, with only one side of the MK sequence converted in a large amount of its product, one half of the LDR probes will be unable to properly hybridize to this sequence and ligation will not occur. LDR detection will only reveal the lesser quantity of fully converted template. Nevertheless, the amount of MK product is greater than the control in these two lanes. Compare Figure 13, lane 8 to Figure 13, lanes 6 and 7. While formamide may reduce conversion efficiency, conversion fidelity is greatly improved.

Newton et al., "Analysis of Any Point Mutation in DNA. The Amplification Refractory Mutation System (ARMS)," Nucleic Acids Res., 17(7):2503-16 (1989) found that C • T, A • A, and T • T mismatches were all far more difficult to extend with *Taq* polymerase than purine-pyrimidine mismatches. These results reflect PCR efficiency of extension rather than fidelity. Others have observed low fidelity in extending natural base mismatches (O'Dell et al., Genome Res., 6(6):558-68 (1996); and Eiken et al., "Application of Natural and Amplification Created Restriction Sites for the Diagnosis of PKU Mutations," Nucleic Acids Res., 19(7):1427-30 (1991)). Use of a nucleotide analog with structural similarities to multiple bases could potentially be used to allow polymerase extension (reading) from the analog when paired with different bases and insertion of different bases opposite the analog (writing). For the purposes of the present invention, the efficiency of the preconversion process need not necessarily be high. However, successful conversion requires high PCR fidelity to ensure that only the bases targeted for conversion are altered. False-positive mutation artifacts will result from alterations of bases not targeted for conversion within the sequence probed for mutations. Preconversion using 3' Q₆ primers

5 forming a Q₆:G mismatch avoids starting polymerase extension with a G • T mismatch. In subsequent amplification cycles, A is apparently written frequently
10 opposite Q₆. This observation is consistent with the results of Hill et al. in which Q₆ base-paired like C and T with nearly equal frequency (Hill et al., "Polymerase
5 Recognition of Synthetic Oligodeoxyribonucleotides Incorporating Degenerate Pyrimidine and Purine Bases," Proc. Natl. Acad. Sci. USA, 95(8):4258-63
15 (1998)). Facile tautomerization allows Q₆ to mimic either pyrimidine when base-paired and avoids mismatch wobble. When the natural base primer is added after
20 preconversion, a significant quantity of perfect match template already exists, otherwise the MK artifact would appear in the reaction regardless of
preconversion. Other nucleotide analogs in addition to Q₆ may serve as a bridge for more efficient conversions (Day et al., Nucleic Acids Res., (1999)).

The fidelity of polymerase extension from primers in PCR has been
25 measured, and conditions were found, which in some cases improve fidelity.

15 Presumably, higher fidelity resulted from a decrease in polymerase misincorporation, primer slippage, and template degradation. PCR/RE/LDR
allows the measurement of very low-level "mutant" sequences by preferentially
30 amplifying non-wild-type sequences. The method of the present invention clearly demonstrates that natural base mismatch primer extension cannot be used as a
20 general technique to create restriction sites at will in any sequence for RFLP analysis. As shown in Figure 13 and observed previously (O'Dell et al., Genome
35 Res., 6(6):558-68 (1996); and Eiken et al., "Application of Natural and Amplification Created Restriction Sites for the Diagnosis of PKU Mutations,"
Nucleic Acids Res., 19(7):1427-30 (1991)), natural base mismatch extension is
40 prone to error. To perfectly engineer a restriction site from existing sequence, an error-free approach is required. The results of these examples, using the method
of the present invention, indicates that the use of nucleotide analogs combined
45 with high fidelity PCR conditions may radically decrease errors. Monitoring the true specificity of primer extension was possible in these studies because LDR can
30 measure specific PCR errors accurately and with high sensitivity. Thus, the products of different polymerases and buffers could be assayed at different steps
50 during PCR/RE/LDR to maximize both PCR efficiency and fidelity. As a result, a PCR/RE/LDR strategy could be assembled to achieve the goal of 10⁵ sensitivity.

5 However, this highest sensitivity was achieved only in the special case of no
conversion at a preexisting *MspI* site. At this time, primer slippage remains an
important mechanism through which mismatch primer extension errors can arise
10 (Day et al., Nucleic Acids Res., (1999)). Although the importance of this source
of error *in vivo* is uncertain, it may have a dramatic impact on allele-specific PCR
5 and other *in vitro* methods of mutation detection. An additional source of error
arises from using natural base primers to select specific sequences for
15 amplification following preconversion with nucleotide analogs. This is because a
fraction of the selective natural base primers may form a mismatched pair with
bases other than the intended base. It is known that a characteristic set of different
20 bases insert opposite nucleotide analogs (Day et al., Nucleic Acids Res., (1999);
and Hill et al., "Polymerase Recognition of Synthetic Oligodeoxyribonucleotides
Incorporating Degenerate Pyrimidine and Purine Bases," Proc. Natl. Acad. Sci.
25 USA, 95(8):4258-63 (1998)). Thus, a high fidelity mismatch primer extension
protocol awaits the development of new convertides that can overcome these
15 problems. In combination with high fidelity PCR and LDR monitoring of
efficiency, mismatch primer extension may become a technique for the precise
30 introduction of desired mutations without artifacts.

Although the invention has been described in detail for the purpose
20 of illustration, it is understood that such details are solely for that purpose. The
variations can be made therein by those skilled in the art without departing from
35 the spirit of the scope of the invention which is defined by the following claims.



Claims

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WHAT IS CLAIMED:

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1. A method for identifying one or more low abundance sequences differing by one or more single-base changes, insertions, or deletions, from a high abundance sequence in a plurality of target nucleotide sequences

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5 comprising:

providing a sample potentially containing one or more low abundance target nucleotide sequences with at least one sequence difference each from the high abundance target sequences;

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providing a primary oligonucleotide primer set characterized by (a) a first oligonucleotide primer containing a target-specific portion, and (b) a second oligonucleotide primer containing a target-specific portion, wherein the primary oligonucleotide primers are suitable for hybridization on complementary strands of a corresponding high and low abundance target nucleotide sequences to permit formation of a polymerase chain reaction product, but have a mismatch which interferes with formation of such a polymerase chain reaction product when hybridized to any other nucleotide sequence present in the sample;

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providing a polymerase;

blending the sample, the primary oligonucleotide primers, and the polymerase to form a primary polymerase chain reaction mixture;

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20 subjecting the primary polymerase chain reaction mixture to two or more polymerase chain reaction cycles comprising a denaturation treatment, wherein hybridized nucleic acid sequences are separated, a hybridization treatment, wherein the target-specific portions of the primary oligonucleotide primers hybridize to the target nucleotide sequences, and an extension treatment, wherein the hybridized primary oligonucleotide primers are extended to form primary extension products complementary to the target nucleotide sequence to which the primary oligonucleotide primer is hybridized;

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providing a secondary oligonucleotide primer set characterized by (a) a first oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion, and (b) a second oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-

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5 specific portion, wherein the secondary oligonucleotide primers in a particular set
are suitable for hybridization on complementary strands of the primary extension
10 products to permit formation of a secondary polymerase chain reaction product
which contains or creates a restriction endonuclease recognition site when
5 amplifying the high abundance target, but does not contain or create a restriction
endonuclease recognition site when amplifying the one or more low abundance
15 targets;

providing a polymerase;

10 blending the primary extension products, the secondary
oligonucleotide primers, and the polymerase to form a secondary polymerase
20 chain reaction mixture;

subjecting the secondary polymerase chain reaction mixture to two
or more polymerase chain reaction cycles comprising a denaturation treatment,
25 wherein hybridized nucleic acid sequences are separated, a hybridization
treatment, wherein the secondary oligonucleotide primers hybridize to the primary
15 extension products, an extension treatment, wherein the hybridized secondary
oligonucleotide primers are extended to form secondary extension products
30 complementary to the primary extension products, wherein high abundance
secondary extension products contain a restriction site but low abundance
20 secondary extension products do not;

35 providing a restriction endonuclease;

blending the secondary extension product and the restriction
endonuclease to form an endonuclease digestion reaction mixture;

40 subjecting the endonuclease digestion reaction mixture to an
25 endonuclease digestion reaction such that the restriction endonuclease recognizes
and cleaves the restriction endonuclease recognition site contained within or
created when amplifying the high abundance target but not the low abundance
45 target in the secondary extension products, thus selectively destroying the high
abundance secondary extension products;

30 providing a tertiary oligonucleotide primer set characterized by (a)
a first tertiary primer containing the same sequence as the 5' upstream portion of
50 the first oligonucleotide primer of the secondary oligonucleotide primer set, and
(b) a second tertiary primer containing the same sequence as the 5' upstream

5 portion of a second oligonucleotide primer of the secondary oligonucleotide primer set, wherein the set of tertiary oligonucleotide primers may be used to
10 amplify all of the secondary extension products;

blending the secondary extension products, the tertiary
5 oligonucleotide primer set, and the polymerase to form a tertiary polymerase chain reaction mixture;

15 subjecting the tertiary polymerase chain reaction mixture to two or more polymerase chain reaction cycles comprising a denaturation treatment, wherein hybridized nucleic acid sequences are separated, a hybridization
20 treatment, wherein the tertiary oligonucleotide primers hybridize to the secondary extension products, an extension treatment, wherein the hybridized tertiary oligonucleotide primers are extended to form tertiary extension products complementary to the secondary extension products;

25 providing a plurality of oligonucleotide probe sets, each set characterized by (a) a first oligonucleotide probe, having a tertiary extension product-specific portion and a detectable reporter label, and (b) a second
30 oligonucleotide probe, having a tertiary extension product-specific portion, wherein the oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a complementary tertiary
20 extension product-specific portion, but have a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence present in the
35 sample;

providing a ligase;

40 blending the tertiary extension product, the plurality of oligonucleotide probe sets, and the ligase to form a ligase detection reaction mixture;

45 subjecting the ligase detection reaction mixture to one or more ligase detection reaction cycles comprising a denaturation treatment, wherein any hybridized oligonucleotides are separated from the tertiary extension products,
30 and a hybridization treatment, wherein the oligonucleotide probe sets hybridize at adjacent positions in a base-specific manner to their respective tertiary extension products, if present, and ligate to one another to form a ligation product sequence containing (a) the detectable reporter label and (b) the tertiary extension product-

5 specific portions connected together, wherein the oligonucleotide probe sets may
hybridize to nucleotide sequences other than their respective complementary
10 tertiary extension products but do not ligate together due to a presence of one or
more mismatches and individually separate during the denaturation treatment; and
5 detecting the reporter labels of the ligation product sequences.
thereby indicating the presence of one or more low abundance target nucleotide
15 equences in the sample.

2. A method according to claim 1, wherein the oligonucleotide
10 probes in a particular oligonucleotide probe set have a unique length so that the
ligation product sequences which they form can be distinguished from other
nucleic acids:
separating the ligation product sequences by electrophoretic
25 mobility prior to said detecting and
15 distinguishing, after said detecting, the ligation product sequences
which differ in electrophoretic mobility.

3. A method according to claim 1, wherein the second
oligonucleotide probe of each oligonucleotide probe set has an addressable array-
20 specific portion, said method further comprising:
providing a solid support with different capture oligonucleotides
35 immobilized at different particular sites, wherein the capture oligonucleotides
have nucleotide sequences complementary to the addressable array-specific
portions and
40 25 contacting the ligase detection reaction mixture, after said
subjecting it to one or more ligase detection reaction cycles, with the solid support
under conditions effective to hybridize the ligation product sequences to the
capture oligonucleotides in a base-specific manner, thereby capturing the
45 addressable array-specific portions to the solid support at the site with the
30 complementary capture oligonucleotide, wherein said detecting indicates the
presence of ligation product sequences captured using the addressable array-
specific portions and immobilized to the solid support at particular sites, thereby
50 indicating the presence of one or more target nucleotide sequences in the sample.

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4. A method according to claim 1, wherein the relative amounts of one or more of a low abundance sequence, differing by one or more single-base changes, insertions, deletions, or translocations and present in a sample in unknown amounts with one or more high abundance sequence in a plurality of target nucleotides, are quantified, said method further comprising:
- quantifying, after said subjecting the primary polymerase chain reaction mixture to one or more polymerase chain reaction cycles, the amounts of primary extension products;
- providing a known amount of one or more marker target nucleotide sequences;
- providing one or more sequence-specific probe sets, including probe sets specifically designed for the marker target nucleotide sequences;
- blending the marker target nucleotide sequences, and the probe sets specifically designed for the marker target nucleotide sequences with ligase detection reaction mixture;
- quantifying the amount of ligation product sequences; and
- comparing the amount of ligation product sequences generated from the unknown low abundance sample with the amount of ligation product sequences generated from known amounts of marker target nucleotide sequences to provide a quantitative measure of the relative level of one or more low abundance target nucleotide sequences in the sample.
5. A method according to claim 4, wherein the relative amounts of one or more of a low abundance sequence, differing by one or more single-base changes, insertions, deletions, or translocations and present in a sample in unknown amounts is present in less than a 1:1,000 molar ratio to the amount of the high abundance sequence in a plurality of target nucleotides.
6. A method according to claim 4, wherein the relative amounts of one or more of a low abundance sequence, differing by one or more single-base changes, insertions, deletions, or translocations and present in a

sample in unknown amounts is present in less than a 1:10,000 molar ratio to the amount of the high abundance sequence in the sample.

7. A method according to claim 4, wherein the relative amounts of one or more of a low abundance sequence, differing by one or more single-base changes, insertions, deletions, or translocations and present in a sample in unknown amounts is present in less than a 1:100,000 molar ratio to the amount of the high abundance sequence in the sample.

8. A method according to claim 1, wherein the efficiency and accuracy of converting the high abundance primary polymerase chain reaction product into a secondary polymerase chain reaction product containing a restriction endonuclease site is improved by performing the following step prior to the providing the secondary oligonucleotide primer set:

providing a pre-secondary oligonucleotide primer set characterized by (a) a first oligonucleotide primer, having a target-specific portion, and (b) a second oligonucleotide primer, having a target-specific portion, wherein the target-specific portions are identical or substantially identical to the secondary oligonucleotide primer set but at least one primer contains one or more nucleotide analogs, wherein the oligonucleotide primers in a particular set are suitable for hybridization on complementary strands of the primary extension products to permit formation of a pre-secondary polymerase chain reaction product which contains one or more nucleotide analogs and opposite strand base changes, wherein the pre-secondary oligonucleotide primer set facilitates conversion of the primary polymerase chain reaction product sequence into a restriction endonuclease recognition site in the subsequent secondary polymerase chain reaction;

providing a polymerase;

blending the primary extension products, the pre-secondary oligonucleotide primers, and the polymerase to form a pre-secondary polymerase chain reaction mixture;

subjecting the secondary polymerase chain reaction mixture to two or more polymerase chain reaction cycles comprising a denaturation treatment,

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wherein hybridized nucleic acid sequences are separated, a hybridization treatment, wherein the secondary oligonucleotide primers hybridize to the primary extension products, an extension treatment, wherein the hybridized secondary oligonucleotide primers are extended to form pre-secondary extension products complementary to the primary extension products, wherein the pre-secondary extension products contain one or more nucleotide analogues and opposite strand base changes which facilitate conversion of the primary polymerase chain reaction product sequence into a restriction endonuclease recognition site in the subsequent secondary polymerase chain reaction, wherein the pre-secondary extension products are then used in place of the primary extension products in the secondary polymerase chain reaction mixture.

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9. A method according to claim 8, wherein the oligonucleotide probes in a particular oligonucleotide probe set have a unique length so that the ligation product sequences which they form can be distinguished from other nucleic acids:

separating the ligation product sequences by electrophoretic mobility prior to said detecting and distinguishing, after said detecting, the ligation product sequences which differ in electrophoretic mobility.

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10. A method according to claim 8, wherein the second oligonucleotide probe of each set has an addressable array-specific portion, said method further comprising:

providing a solid support with different capture oligonucleotides immobilized at different particular sites, wherein the capture oligonucleotides have nucleotide sequences complementary to the addressable array-specific portions and

contacting the ligase detection reaction mixture, after said subjecting it to one or more ligase detection reaction cycles, with the solid support under conditions effective to hybridize the ligation product sequences to the capture oligonucleotides in a base-specific manner, thereby capturing the addressable array-specific portions to the solid support at the site with the

complementary capture oligonucleotide, wherein said detecting indicates the presence of ligation product sequences captured using the addressable array-specific portions and immobilized to the solid support at particular sites, thereby indicating the presence of one or more target nucleotide sequences in the sample.

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11. A method according to claim 8, wherein the relative amounts of one or more of a low abundance sequence, differing by one or more single-base changes, insertions, deletions, or translocations and present in a sample in unknown amounts with one or more high abundance sequence in a plurality of target nucleotide, are quantified, said method further comprising:

quantifying, after said subjecting the primary polymerase chain reaction mixture to one or more polymerase chain reaction cycles, the amounts of primary extension products;

providing a known amount of one or more marker target nucleotide sequences;

providing one or more sequence-specific probe sets, including probe sets specifically designed for the marker target nucleotide sequences;

blending the marker target nucleotide sequences, and the probe sets specifically designed for the marker target nucleotide sequences with ligase detection reaction mixture;

quantifying the amount of ligation product sequences; and

comparing the amount of ligation product sequences generated from the unknown low abundance sample with the amount of ligation product sequences generated from known amounts of marker target nucleotide sequences to provide a quantitative measure of the relative level of one or more low abundance target nucleotide sequences in the sample.

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13. A method according to claim 11, wherein the relative amounts of one or more of a low abundance sequence, differing by one or more single-base changes, insertions, deletions, or translocations and present in a sample in unknown amounts is present in a molar ratio of less than 1:10,000 to the amount of the high abundance sequence in the sample.

14. A method according to claim 11, wherein the relative amounts of one or more of a low abundance sequence, differing by one or more single-base changes, insertions, deletions, or translocations and present in a sample in unknown amounts is present in a ratio molar ratio of less than 1:100,000 to the amount of the high abundance sequence in the sample.

15. A method according to claim 8, where the nucleotide analog of at least one oligonucleotide primer of the pre-secondary oligonucleotide primer set is at the 3' end of the primer.

16. A method according to claim 8, where the nucleotide analog is selected from the group consisting of 1-(2'-deoxy-β-D-ribofuranosyl)imidazole-4-carboxamide, 1-(2'-deoxy-β-D-ribofuranosyl)-3-nitropyrrole, 2'-deoxyinosine, 6-(2'-deoxy-β-D-ribofuranosyl)-6II,8II-3,4-dihydropyrimido[4,5-c][1,2]oxazine-7-one, 2-amino-7-(2'-deoxy-β-D-ribofuranosyl)-6-methoxyaminopurine, 1-(2'-deoxy-β-D-ribofuranosyl)-4-iodopyrazole, 1-(2'-deoxy-β-D-ribofuranosyl)pyrrole-3-carboxamide, and 1-(2'-deoxy-β-D-ribofuranosyl)-4-nitropyrazole.

17. A method according to claim 1 further comprising:
repeating the endonuclease digestion reaction after said subjecting the tertiary polymerase chain reaction mixture to two or more polymerase chain reaction cycles and after said subjecting the ligase detection reaction mixture to one or more ligase detection reaction cycles, wherein, during said repeating the endonuclease digestion reaction, the restriction endonuclease recognizes and cleaves the restriction endonuclease recognition site contained within any

remaining high abundance target, thereby selectively destroying the high abundance tertiary extension products.

18. A kit for identifying one or more low abundance sequences differing by one or more single-base changes, insertions, or deletions, from a high abundance sequence in a plurality of target nucleotide sequences comprising:
- providing a primary oligonucleotide primer set characterized by (a) a first oligonucleotide primer containing a target-specific portion, and (b) a second oligonucleotide primer containing a target-specific portion, wherein the primary oligonucleotide primers are suitable for hybridization on complementary strands of a corresponding high and low abundance target nucleotide sequences to permit formation of a primary extension product, but have a mismatch which interferes with formation of such a polymerase chain reaction product when hybridized to any other nucleotide sequence present in the sample;
 - providing a secondary oligonucleotide primer set characterized by (a) a first oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion, and (b) a second oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion, wherein the secondary oligonucleotide primers in a particular set are suitable for hybridization on complementary strands of the primary extension products to permit formation of a secondary extension product which contains or creates a restriction endonuclease recognition site when amplifying the high abundance target, but does not contain or create a restriction endonuclease recognition site when amplifying the one or more low abundance targets;
 - providing a tertiary oligonucleotide primer set characterized by (a) a first tertiary primer containing the same sequence as the 5' upstream portion of the first oligonucleotide primer of the secondary oligonucleotide primer set, and (b) a second tertiary primer containing the same sequence as the 5' upstream portion of a second oligonucleotide primer of the secondary oligonucleotide primer set, wherein the set of tertiary oligonucleotide primers may be used to amplify all of the secondary extension products; and
 - providing a plurality of oligonucleotide probe sets, each set characterized by (a) a first oligonucleotide probe, having a tertiary extension

5 product-specific portion and a detectable reporter label, and (b) a second
oligonucleotide probe, having a tertiary extension product-specific portion,
10 wherein the oligonucleotide probes in a particular set are suitable for ligation
together when hybridized adjacent to one another on a complementary tertiary
5 extension product-specific portion, but have a mismatch which interferes with
such ligation when hybridized to any other nucleotide sequence present in the
15 sample.

19. A kit according to claim 18 further comprising:
20 providing a pre-secondary oligonucleotide primer set characterized
by (a) a first oligonucleotide primer, having a target-specific portion, and (b) a
second oligonucleotide primer, having a target-specific portion, wherein the
25 target-specific portions are identical or substantially identical to the secondary
oligonucleotide primer set but at least one primer contains one or more nucleotide
15 analogs, wherein the pre-secondary oligonucleotide primers in a particular set are
suitable for hybridization on complementary strands of the primary extension
products to permit formation of a pre-secondary extension product which contains
30 one or more nucleotide analogs and opposite strand base changes, wherein the
pre-secondary oligonucleotide primer set facilitates conversion of the primary
20 extension product sequence into a restriction endonuclease recognition site in a
subsequent secondary polymerase chain reaction.

20. A kit according to claim 18 further comprising:
40 a ligase.

21. A kit according to claim 18 further comprising:
45 a polymerase.

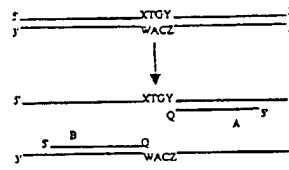
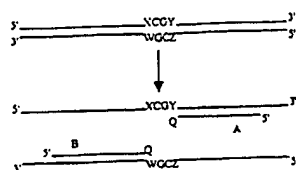
22. A kit according to claim 18 further comprising:
30 a restriction endonuclease.

STEPS IN
PCR/RE/LDR

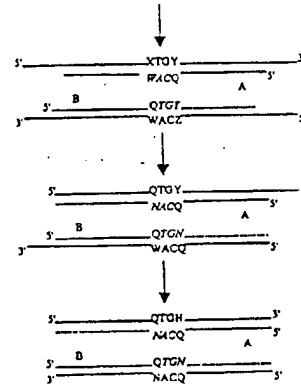
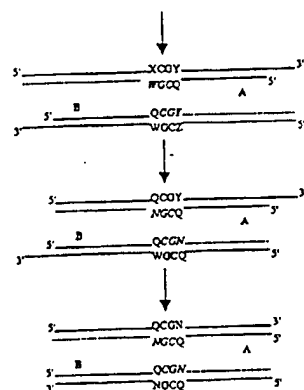
WILD-TYPE 1 / 15

MUTANT

1)



2)



3)

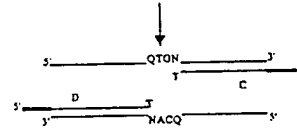
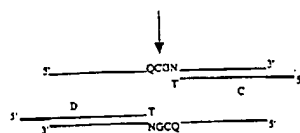
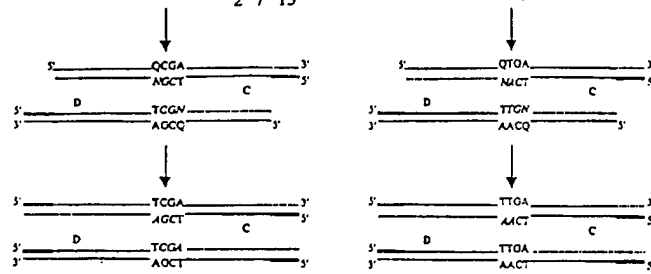
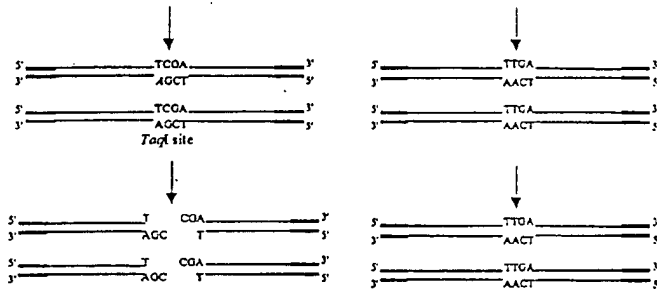


FIGURE 1

4)



5)



6)

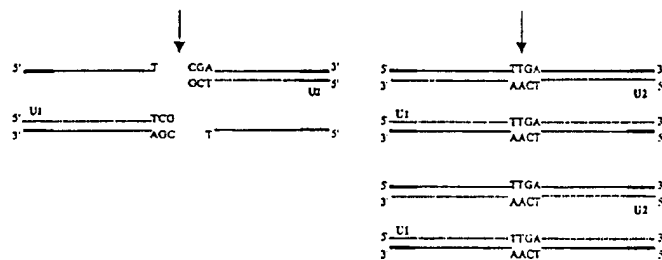
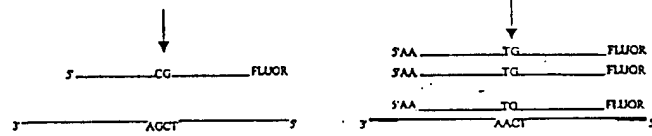
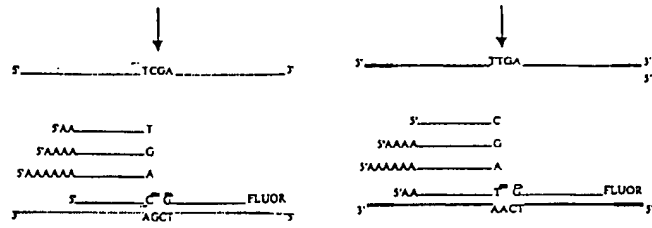


FIGURE 1 (Cont'd.)

7A)



From 6)

From 6)

7B)

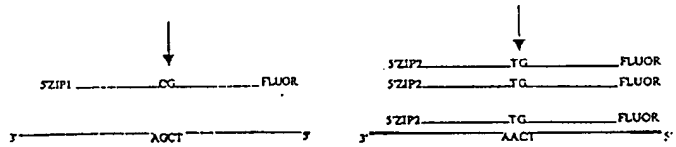
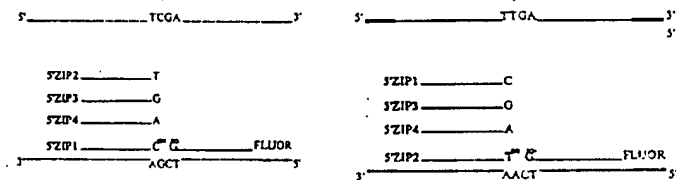


FIGURE 1 (Cont'd.)

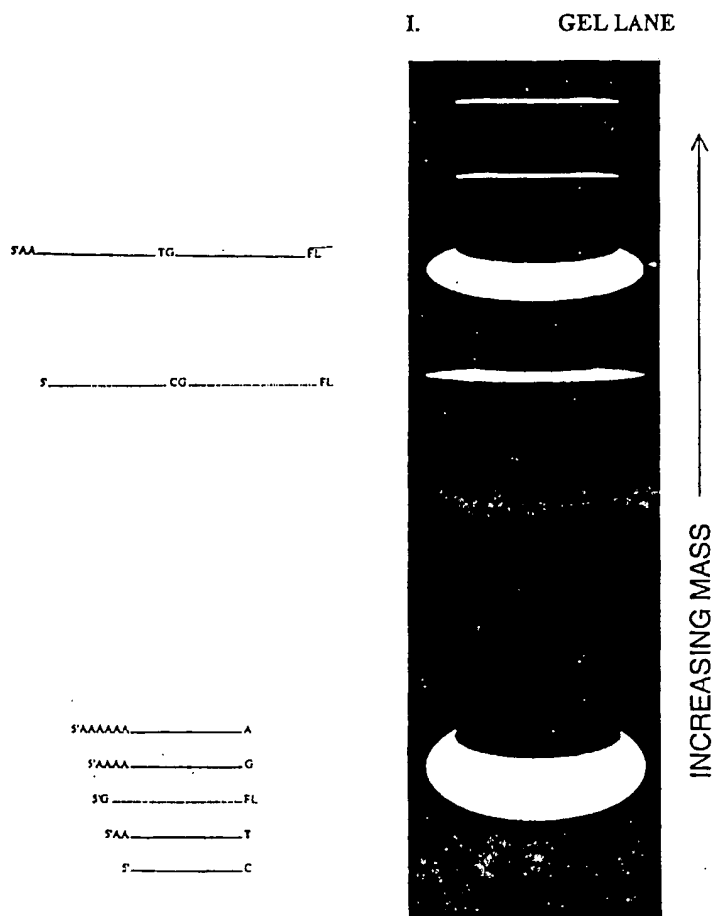


FIGURE 2

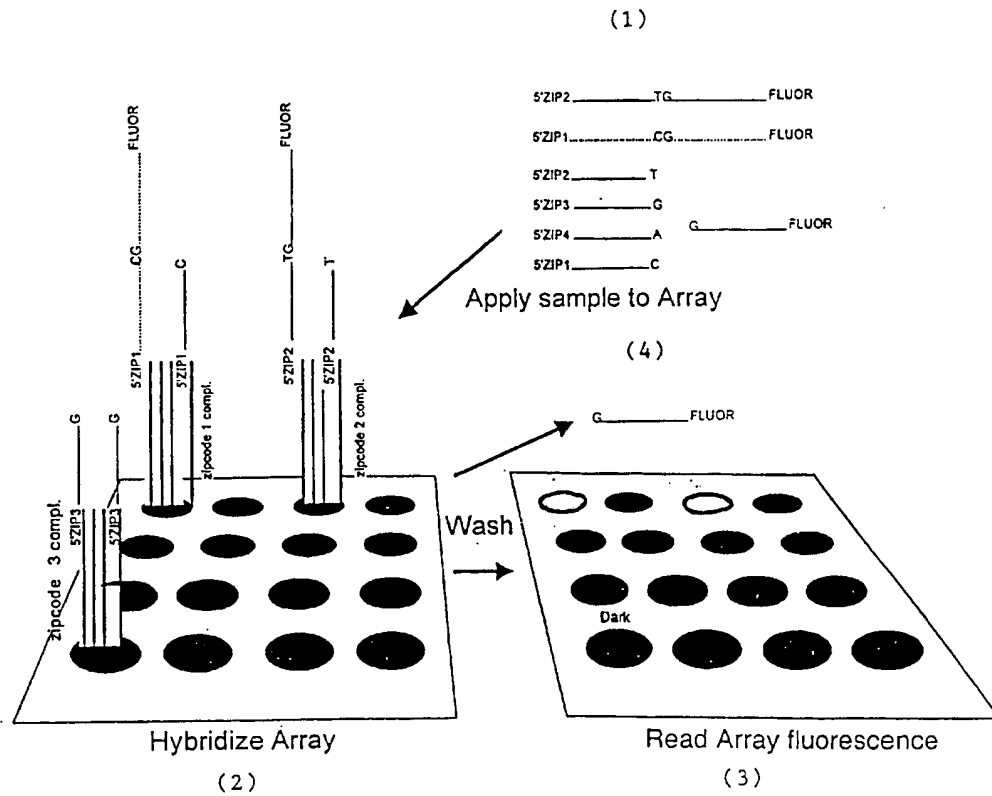


FIGURE 3

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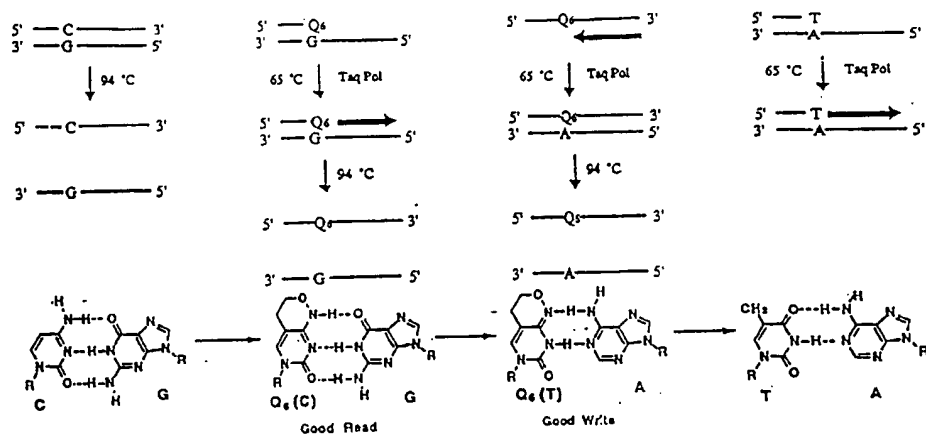


FIGURE 4

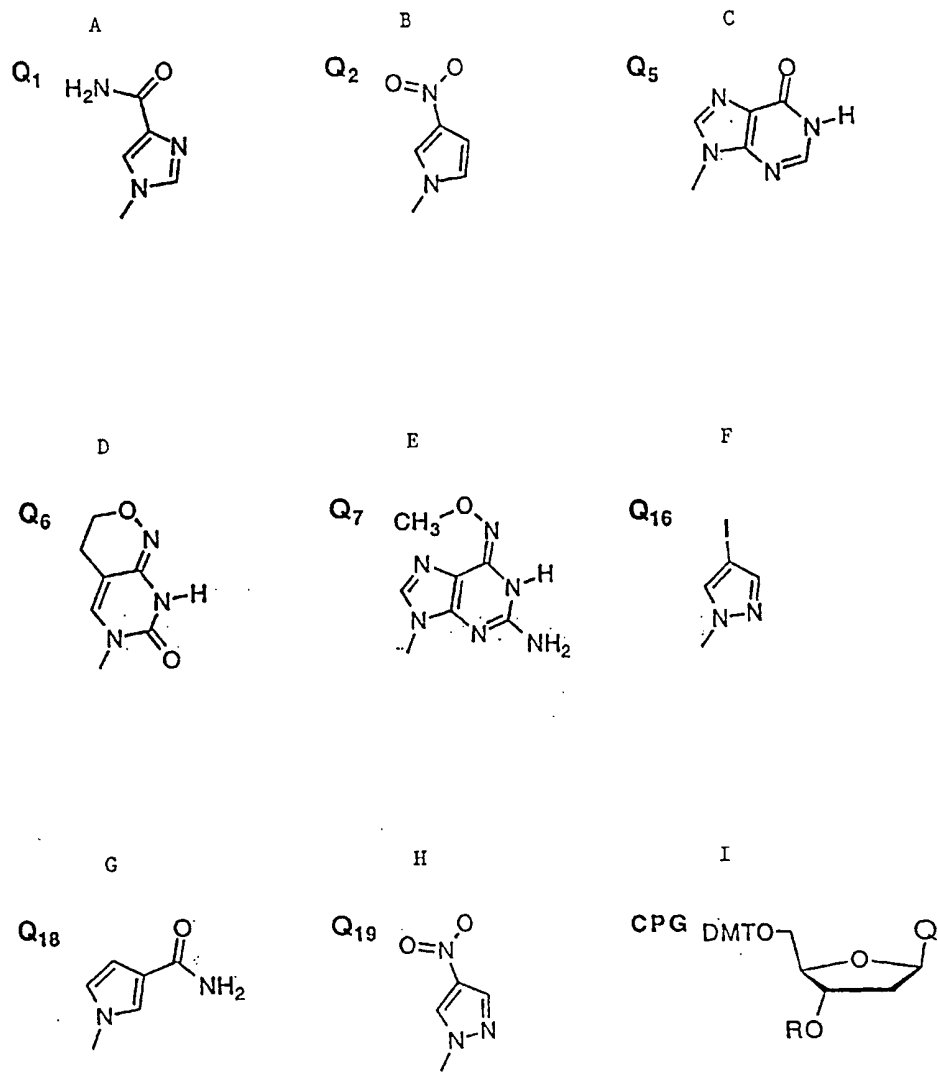


FIGURE 5

A

Primers

Ztop

p53zlp248

p53-248X

50 bp synthetic
duplex DNA

p53-248XR

p53zlp248R

Zbot

B

Primers

Ztop

p53zlp248T

p53-248Q_H50 bp synthetic
duplex DNAp53-248Q_RR

p53zlp248TR

Zbot

C

LDR Primers

p53LDR248FCA

p53LDR248FCG

p53LDR248FCT

p53LDR248FCC

p53LDR248PGG

conversion
products

Discrimination

Common

F-AAAAAA GC ATG GGC GGC ATG AAC A

F-AAAA GC ATG GGC GGC ATG AAC G

F-AA GC ATG GGC GGC ATG AAC T

F- GC ATG GGC GGC A... AAC G...-ligase

GG AGG GGC ATC CTC ACC ATC AT-block

3' (-strand) ... GTA TGC GCA AGG AGG TAC CCG CCG TAC TTG ACC TCC GGG TAG GAG TCG TAG TAG TGA ACC... 5'

FIGURE 6

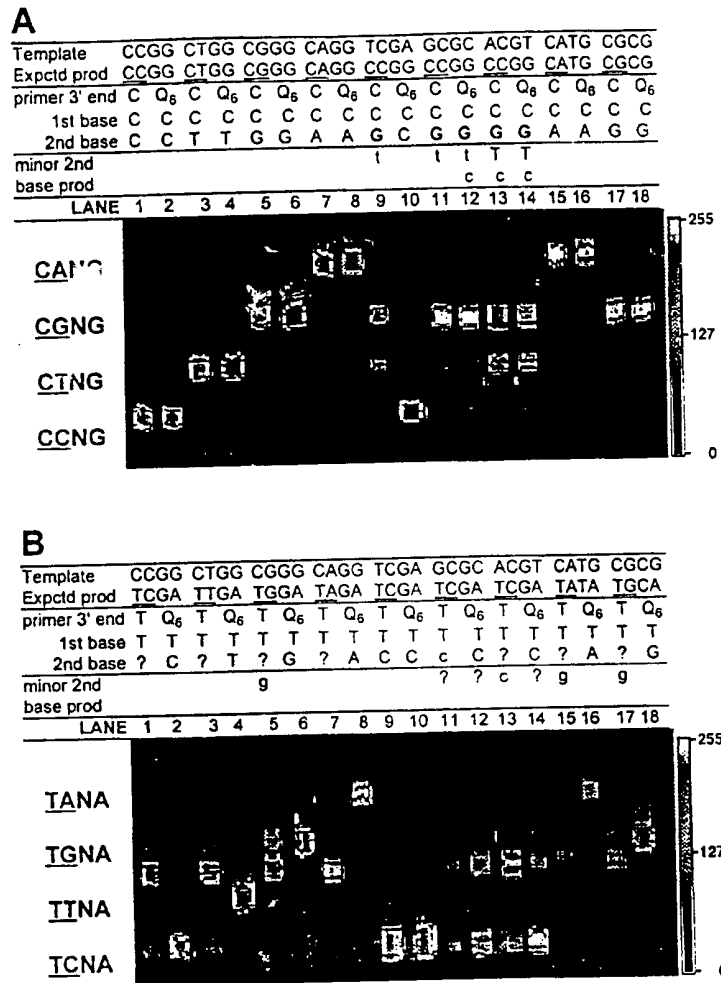
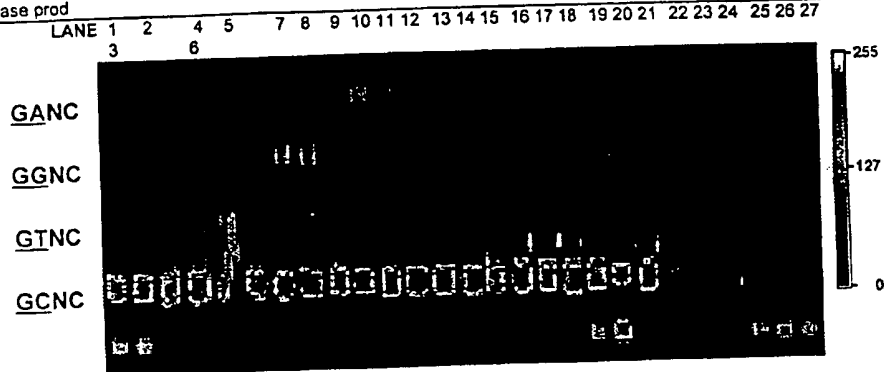


FIGURE 7

A

Template	CCGG	CTGG	CGGG	CAGG	TCGA	GCGC	ACGT	CATG	CGCG
Exptd prod	GCGC	GTGC	GGGC	GAGC	GCGC	GCGC	GCGC	GATC	GGCC
primer 3' end	G Q ₅ Q ₇	G Q ₅ Q ₇	G Q ₅ Q ₇	G Q ₅ Q ₇	G Q ₅ Q ₇	G Q ₅ Q ₇	G Q ₅ Q ₇	G Q ₅ Q ₇	G Q ₅ Q ₇
1st base	G G G	G G G	G G G	G G G	G G G	G G G	G G G	G G G	G G G
2nd base	C C C	C C C	C C C	C C C	C C C	C C C	C C C	C C C	? ? ?
minor 2nd base prod		t t t	g g	A A a					

**B**

Template	CCGG	CTGG	CGGG	CAGG	TCGA	GCGC	ACGT	CATG	CGCG
Exptd prod	ACGT	ATGT	AGGT	AAGT	ACGT	ACGT	ACGT	AATT	AGCT
primer 3' end	A Q ₇ Q ₅	A Q ₇ Q ₅	A Q ₇ Q ₅	A Q ₇ Q ₅	A Q ₇ Q ₅	A Q ₇ Q ₅	A Q ₇ Q ₅	A Q ₇ Q ₅	A Q ₇ Q ₅
1st base	A A A	A A A	A A A	A A A	A A A	A A A	A A A	A A A	A A A
2nd base	C C C	C C C	C C C	C C C	C C C	C C C	C C C	C C C	C C C
minor 2nd base prod	?		?						

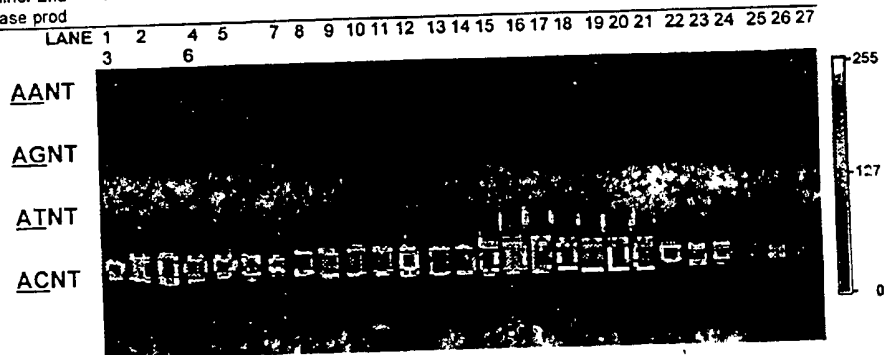


FIGURE 8

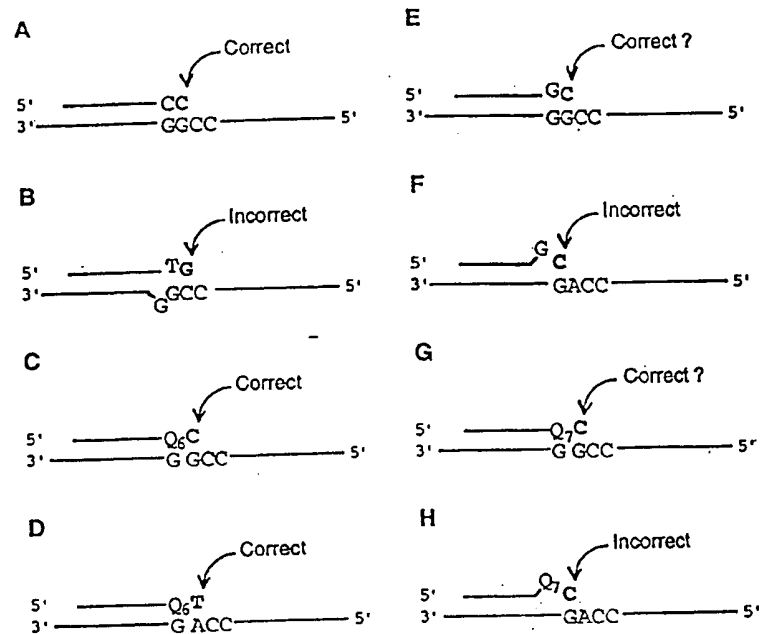


FIGURE 9

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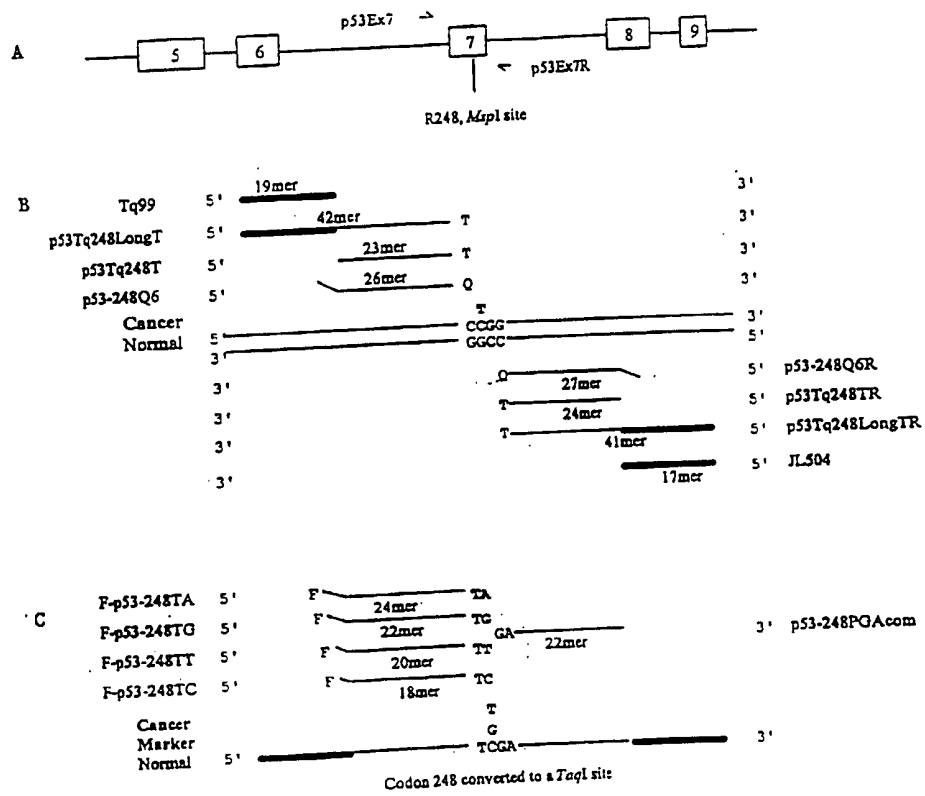


FIGURE 10

A

Primers
 Zlop CTT GCA GCA GTT CAT AAG C
 p53zp248short CTT GCA GCA GTT CAT AAG CTT TCC TCC ATG GGC GGC ATG A
 p53-248short GT TCC TCC ATG GGC GGC A→pdl
 p53 exon 7 3'...CA AAG AAG TAC CCG CCG TAC TTT TCC TCC GGG TAG CAG TGG TAG TAG TGT... 5' (-)
 PCR product 5'...GT TCC TCC ATG GGC GGC ATG AAC GGG AAG GGC ATC CTC ACC ATC ATC ACA... 3' (+)
 (MK not shown) III III III III III III III
 p53-248shortR pol-α TAG CAG TGG TAG TAG TO
 p53zp248shortR C GGG TAG CAG TGG TAG TAG TCC ACC GCT GCG TCA AAC C
 Zbot C AAG GCT GCG TCA AAC C

B

Primers
 Zlop CTT GCA GCA GTT CAT AAG C
 p53zp248T CTT GCA GCA GTT CAT AAG CTT TCC TCC ATG GGC GGC ATG AAT
 p53Taq248T GT TCC TCC ATG GGC GGC ATG AAT
 p53Taq248Q₆ TTCT TCC TCC ATG GGC GGC ATG AAG→pdl
 60 bp synthetic duplex DNA, or PCR product III III III III III III III
 p53Taq248Q₆R 3' CA AAG AAG TAC CCG CCG TAC TTT GGC TCC GGG TAG CAG TGG TAG TAG TGT 5' (-)
 5' GT TCC TCC ATG GGC GGC ATG AAC GGG AAG GGC ATC CTC ACC ATC ATC ACA... 3' (+)
 III III III III III III III
 p53Taq248TR pol-α TAG CAG TGG TAG TAG TCTT
 p53zp248TR T TCC GGG TAG CAG TGG TAG TAG TO
 Zbot T TCC GGG TAG CAG TGG TAG TAG TCC ACC GCT GCG TCA AAC C
 C AAG GCT GCG TCA AAC C

C

LDR Primers
 p53LDR248FTCL F-AAAAAAAA GC ATG GGC GGC ATG AAT C
 p53LDR248FCA F-AAAAA GC ATG GGC GGC ATG AAC A
 p53LDR248FCG F-AAAA GC ATG GGC GGC ATG AAC G
 p53LDR248FCT F-AA GC ATG GGC GGC ATG AAC T
 p53LDR248FOC F- GC ATG GGC GGC ATG AAC C *ligase*
 p53LDR248PGG OG AAG CCC ATC CTC ACC ATC AT-block
 conversion products 3' (-strand) III III III III III III III III III III III III III III III
 ... GTA TCC GCA AAG AAG TAC CCG CCG TAC TTT TCC TCC GGG TAG CAG TGG TAG TAG TGA ACC... 5'

FIGURE 11

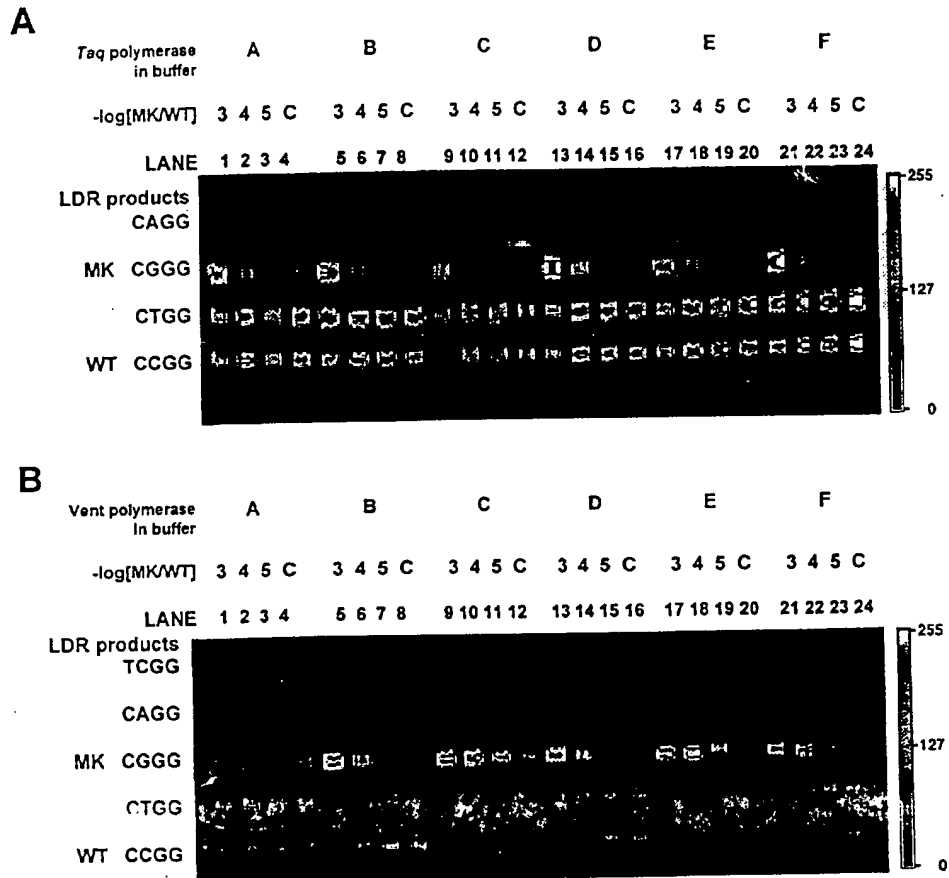


FIGURE 12

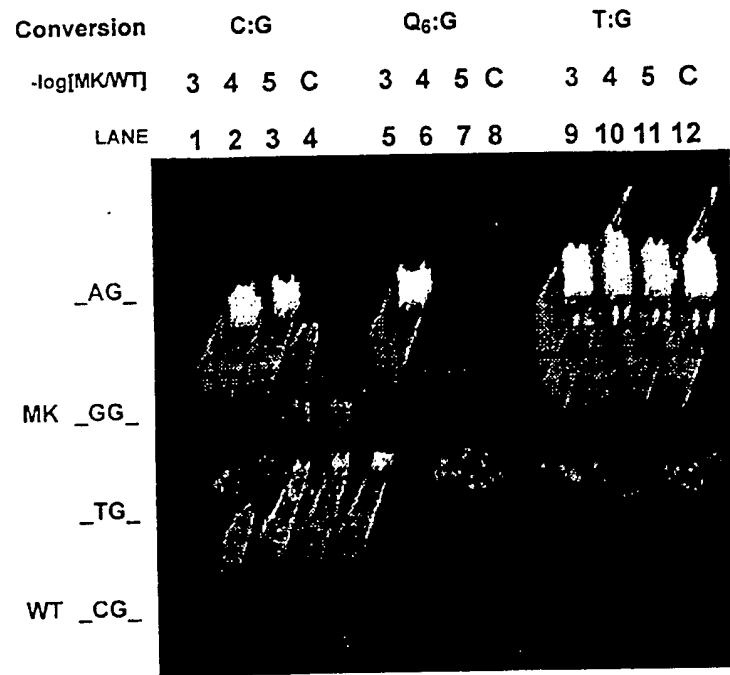


FIGURE 13

SEQUENCE LISTING

<110> Cornell Research Foundation, Inc. et al.

<120> COUPLED POLYMERASE CHAIN REACTION-RESTRICTION
ENDONUCLEASE DIGESTION-LIGASE DETECTION REACTION
PROCESS

<130> 19603/482

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<151> 1999-03-19

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<170> PatentIn Ver. 2.1

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Oligonucleotide Primer

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Oligonucleotide Primer

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